Tesis doctoral:

Epidemiología y diagnóstico de la amphimeriosis en Ecuador.

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Certificación:

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Que la Tesis Doctoral titulada ***"Epidemiología y Diagnóstico de la Amphimeriosis en Ecuador"*,** que se presenta para optar al grado de Doctor por la Universidad de Salamanca en la modalidad de *Tesis por compendio de publicaciones*, ha sido realizada por **William Fernando Cevallos Trujillo,** con CC. No. 170989629-2, Médico por la Universidad Central del Ecuador y Magister en Medicina Tropical por la Fundación Oswaldo Cruz de Rio de Janeiro-Brasil, bajo nuestra dirección en el Centro de Biomedicina de la Universidad Central del Ecuador y en el Centro de Investigación de Enfermedades Tropicales de la Universidad de Salamanca, dentro del Programa de Doctorado Salud y Desarrollo en los Trópicos. Consideramos que reúne, a nuestro juicio, originalidad y contenidos suficientes, por lo que autorizamos su presentación para ser evaluada.

Y para que así conste, a efectos legales, expiden el presente certificado en Salamanca, a 20 de Febrero de 2018.

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Dedicatoria

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*"**Per aspera, ad astra**"*
"Por el sendero áspero, a las estrellas"
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B. Índice de figuras

C. Índice de tablas

CONTENIDO

1. INTRODUCCIÓN

1.1 Introducción

El ser humano se ve constantemente amenazado por antiguos y nuevos agentes patógenos, entre ellos los parásitos trematodos causantes de un sinnúmero de enfermedades. Estos parásitos tienen complejos ciclos de vida que incluyen hospedadores intermediarios, definitivos y reservorios en un intrincado y complejo mecanismo de adaptación y supervivencia.

Las regiones tropicales y subtropicales de países pobres, son las más propicias para el desarrollo de ciertas parasitosis humanas, ya que cuentan con un ambiente natural y social que les son favorables para su cadena reproductiva.

Los adelantos científicos y tecnológicos en el conocimiento de estas enfermedades, no siempre van de la mano con las mejoras en el ambiente, el saneamiento e higiene que permitan interrumpir los ciclos de transmisión de las parasitosis humanas.

El desarrollo de nuevos métodos diagnósticos se hace imperioso para conocer la real carga de infección y enfermedad que los parásitos producen, así como para descubrir nuevos agentes infecciosos que pudiendo estar en otros hospedadores no humanos pueden infectar a éstos. Es así como, a partir del descubrimiento de la infección en humanos por el trematodo de vías biliares denominado *Amphimerus* spp. en el año 2011, existe la necesidad de desarrollar métodos diagnósticos sensitivos, específicos, reproducibles y accesibles para establecer su carácter zoonótico y su prevalencia real y distribución geográfica. El único método diagnóstico disponible hasta el momento es el análisis coproparasitario de heces, el cual tiene limitada sensibilidad y especificidad.

Desarrollar nuevos métodos diagnósticos inmunológicos y moleculares es un imperativo para aumentar el conocimiento de esta parasitosis a fin de establecer un diagnóstico oportuno, un tratamiento adecuado y contar con herramientas para la vigilancia epidemiológica en zonas endémicas y no endémicas.

1.2 Enfermedades Emergentes y Re-emergentes

Los Centros de Control de Enfermedades de los Estados Unidos de Norteamérica (CDC; del inglés, Centers for Disease Control and Prevention), define como enfermedades emergentes a aquellas nuevas infecciones producidas por nuevos patógenos desconocidos o enfermedades infecciosas conocidas las cuales se descubren en nuevas áreas geográficas. Enfermedades re-emergentes serían aquellas infecciones que aumentan o amenazan incrementarse con el tiempo, como consecuencia de la resistencia a los medicamentos o a una falta de control apropiado derivado de las políticas sanitarias de cada país (CDC, 2014). Los CDC, reconocen más de cincuenta Enfermedades Emergentes y Re-emergentes y desde 1990 mediante un “Programa de

Infecciones Emergentes", se ha expandido por todo el mundo (CDC, 2014 a; CDC 2014b; Breiman et al., 2013).

De acuerdo con la Organización Mundial de la Salud (OMS), cada año aparecen en el mundo nuevas enfermedades infecciosas (WHO, 2007). La mayoría se consideran zoonosis, siendo más de tres cuartas partes originadas en animales silvestres (Jones et al., 2008). Aproximadamente, el 64% de las enfermedades infecciosas que afectan a las personas, son causadas por patógenos que se transmiten entre diferentes especies de animales domésticos y silvestres (Heeney, 2006; Davis, 2008; Taylor et al., 2001).

Esta tendencia, ha dado lugar a un enfoque más holístico e integrado de la salud humana, animal y ambiental, conocida como "Una Salud", la misma que viene siendo impulsada por Naciones Unidas (Pappaioanou et al., 2008). Esta propuesta ha merecido especial atención en las últimas décadas, buscando una estrategia global para mejorar el diagnóstico, control y tratamiento de estas enfermedades (Kaplan et al., 2009; Heymann et al., 2014; Mackey et al., 2012). Este nuevo paradigma, ha sido aplicado, por ejemplo, mejorando el diagnóstico en las trematodosis zoonóticas desatendidas (Johansen et al., 2015).

En un mundo cada vez más globalizado e interconectado, nuevos y viejos parásitos amenazan la salud de las poblaciones humanas de otros continentes a los de su origen. La globalización no solo implica el intercambio de mercancías y tecnología, sino también de patógenos causantes de enfermedades. Sucediendo esto también a nivel regional e incluso dentro de un país con la migración y el turismo interno, influyendo entre otros el compartir e intercambiar alimentos (Trostle et al., 2008).

Los Institutos Nacionales de Salud de los Estados Unidos (NIH; del inglés, National Institutes of Health), clasifican la aparición de nuevas enfermedades y el resurgimiento de otras, en tres grupos: el grupo 1 corresponde a enfermedades emergentes reconocidas como nuevas en los últimos 20 años, el grupo 2 son las enfermedades re-emergentes propiamente dichas, y, el grupo 3 son las enfermedades producidas por agentes que pueden ser utilizados en acciones bioterroristas (NIAID, 2017).

Esta emergencia o re-emergencia de infecciones nuevas o conocidas, se ve afectada entre otras razones por el acelerado desarrollo humano, incluyendo numerosos cambios demográficos, poblacionales y ambientales, así como el rápido desarrollo de nuevas técnicas de diagnóstico. Estas infecciones se producen sobre todo en países pobres, constituyendo una importante barrera para el desarrollo social y de la salud humana. Debemos resaltar además que no solo existen discrepancias entre países sino también dentro de cada país. Así, hay comunidades que son más afectadas y vulnerables como son las poblaciones indígenas y afrodescendientes, donde las madres y niños,

presentan alta morbimortalidad (Molyneux et al., 2010; WHO, 2013; Baker et al., 2010; Conteh et al., 2010).

1.3 Enfermedades Tropicales Desatendidas.

Existen en el mundo lugares más específicos donde la mayoría de las enfermedades infecciosas emergentes y re-emergentes se desarrollan y atacan, estas son las zonas tropicales y subtropicales. Históricamente, las enfermedades que se presentan en estas zonas de países pobres y en vías de desarrollo son endémicas y continúan desatendidas por los organismos gubernamentales regionales, nacionales e internacionales (WHO, 2010; Liese et al., 2010; Molyneux, 2010). En el año 2007 la OMS reunió a más de 200 expertos para tratar este problema acuñándose el término: "Enfermedades Tropicales Desatendidas" (*Neglected Tropical Diseases* – NTDs por sus siglas en inglés) para denominar estas afecciones (WHO, 2008).

Las NTDs representan la causa más frecuente de enfermedad en 2.7 billones de personas pobres, es decir, aquellas que viven con menos de 2 dólares diarios (Hotez et al., 2010). Estas enfermedades no solo dificultan el desarrollo infantil y causan incapacidad crónica a lo largo de la vida, sino que además impiden el desarrollo económico y social de las poblaciones humanas (Hotez et al., 2006; Fürst et al., 2012).

Son causadas principalmente por virus, bacterias, protozoos y helmintos. Muchas son zoonosis y/o transmitidas por un sinnúmero de artrópodos vectores. Las veinte NTDs que considera la OMS son: dengue, rabia, tracoma, úlcera de Buruli, treponematosis endémica, lepra, enfermedad de Chagas, tripanosomosis Africana humana, leishmaniosis, teniosis/cisticercosis, dracunculosis, equinococosis, trematodosis transmitidas por alimentos, filariosis linfáticas, oncocercosis, esquistosomosis, helmintosis transmitidas por el suelo, pian, mordedura de serpientes, escabiosis y otras ectoparasitosis (Hotez, 2013; WHO, 2017).

La mayoría de las comunidades en las que se presentan estas enfermedades se ven afectadas por limitados recursos gubernamentales tanto humanos como económicos, baja accesibilidad a servicios de salud, escaso saneamiento e higiene, pobreza extrema, nutrición deficiente, afectación de su cohesión social debida a proyectos de desarrollo, prácticas de consumo de alimentos inadecuadas. Muchas de estas enfermedades son prevenibles y/o tratables a través de intervenciones de bajo costo (Eisenberg et al., 2009; WHO, 2013; Baker et al., 2010; Conteh, 2010).

El estigma social, los hábitos culturales, la marginación, la extrema pobreza de las poblaciones afectadas y la baja mortalidad son varios factores que contribuyen en el "olvido" de estas enfermedades. Su prevalencia en zonas geográficas y ambientales específicas fuera del mundo desarrollado y su cuota de mercado insignificante para la

industria farmacéutica reduce aún más la importancia de estas enfermedades en el debate sobre salud global (WHO, 2016). La negligencia u olvido, también es evidente en términos económicos, ya que estas enfermedades reciben una pobre financiación estatal. Así, por ejemplo, durante el periodo 2003-2007, la asignación para proyectos de lucha contra VIH fue del 36,6%, para malaria 3,6% y para tuberculosis 2,2%. Por el contrario, la proporción media para el control de NTDs fue del 0,6% (Liese & Schubert, 2009). La asignación de recursos para la investigación también muestra una asimetría, pues el 80% de recursos se destinan únicamente al abordaje de la malaria, tuberculosis e infección por VIH (Moran et al., 2009).

En América Latina, las NTDs no sólo afectan a la población más pobre, sino que también se concentran en poblaciones vulnerables, especialmente comunidades indígenas y con descendencia africana (Hotez et al., 2008). En esta región del mundo, se estima que el 7% del total de la población y el 40% de la población rural pertenecen a un solo grupo étnico (PAHO, 2007). La extrema pobreza afecta a poblaciones indígenas, particularmente en Bolivia, Colombia, Ecuador, Perú, Guatemala y México, donde reside aproximadamente el 80% de esta población (Holveck et al., 2007).

Las enfermedades zoonóticas desatendidas o *Neglected Zoonosis Diseases* (NZDs), son un subconjunto de las NTDs. De acuerdo a la OMS, 7 de las 20 NTDs, son consideradas enfermedades zoonóticas, a saber: rabia, tripanosomosis africana humana, leishmaniosis, teniosis/cisticercosis, equinococosis, trematodosis transmitidas por alimentos y esquistosomosis (Hotez, 2013; WHO, 2013).

En muchos países del mundo coexisten varias NTDs, lo cual demuestra la complejidad al abordar este tipo de enfermedades (Figura 1).

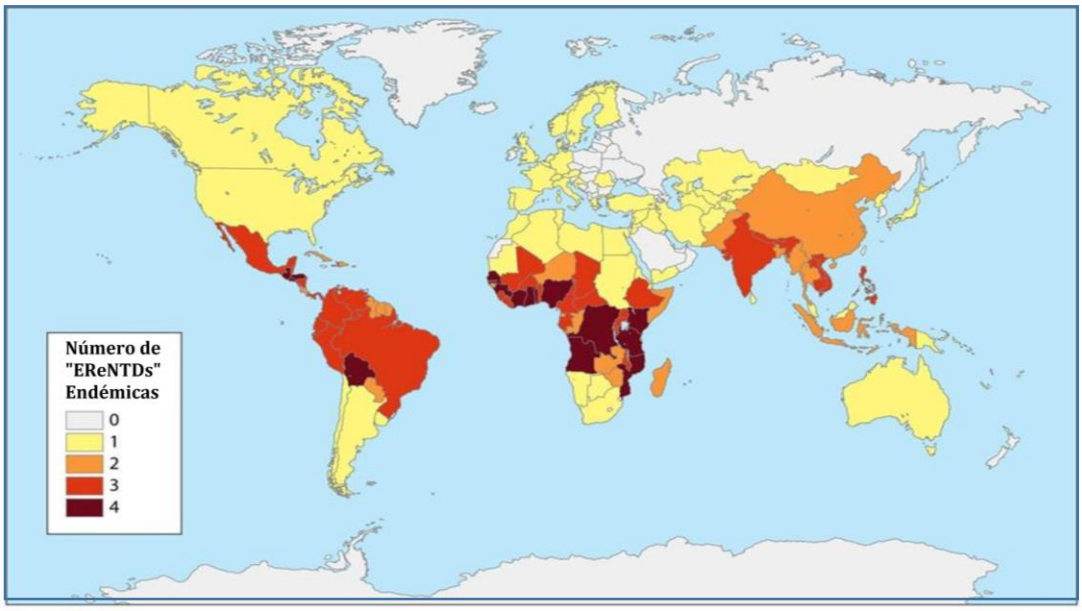


Figura 1. Mapa de intensidad global de la endemicidad por regiones de las Enfermedades Olvidadas Emergentes y Re-emergentes (EReNTDs). Fuente OMS, 2013 (Mackey et al., 2014).

De acuerdo a la clasificación del Banco Mundial, los países que tienen bajos ingresos económicos y que además están ubicados en la línea ecuatorial, aumentan el riesgo para la transmisión y dispersión de las enfermedades zoonóticas (Hotez et al., 2010). Los factores ecológicos y el desarrollo económico, juegan un papel importante en la dinámica de transmisión (McMichael, 2004; Sachs et al., 2001). Así, las regiones alrededor de la línea ecuatorial reciben mayor influencia de los rayos solares, lo que permite una mayor capacidad de supervivencia de las plantas (Thornthwaite, 1948), dando como resultado regiones tropicales con mayor biodiversidad del planeta (Waide et al., 1999). Esto tiene como resultado la presencia de nichos ecológicos propicios para la transmisión de enfermedades infecciosas entre diversos animales (Keesing et al., 2010).

En el Ecuador, las enfermedades tropicales y zoonóticas desatendidas, son un verdadero problema de Salud Pública. Así, la enfermedad de Chagas causada por *T. cruzi* y transmitida por diferentes especies de chinches vectores persiste en amplias zonas tropicales y subtropicales (Dumonteil et al., 2016). El dengue, si bien ha sido considerado una enfermedad urbana, recientemente se ha incrementado en zonas rurales y estaría reemplazando a la malaria como causa de fiebre (Cifuentes et al., 2013).

El control de la rabia canina continúa siendo un desafío en zonas rurales del país donde además se han documentado casos transmitidos por murciélagos (Cartelle Gestal et al., 2015). Uno de los mayores éxitos del Ecuador, ha sido el control y posterior

eliminación a través de la distribución masiva de ivermectina, de la transmisión de *Onchocerca volvulus* causante de la oncocercosis, que afectaba principalmente a pobladores afroecuatorianos y Chachis en el Noroeste del país (Lovato et al., 2014; WHO, 2016). La equinococosis quística causada por *Echinococcus granulosus*, es un problema creciente, posiblemente debido a un mejor diagnóstico a través de métodos más sensibles y a una mejor comprensión de la enfermedad (Cartelle Gestal et al., 2015).

En relación al complejo Teniosis/Cisticercosis, varios estudios en zonas endémicas andinas, han demostrado una alta exposición a las oncosferas de *T. solium* pero baja prevalencia de neurocisticercosis (Rodríguez-Hidalgo et al., 2003; 2006). Otro importante problema de Salud Pública en Ecuador es la leishmaniosis que afecta a 21 de sus 24 provincias especialmente en zonas subtropicales y tropicales, aunque también está presente en zonas de los Andes (Hashiguchi et al., 1991; Calvopiña et al., 2004). Las helmintosis transmitidas por el suelo continúan siendo un importante problema de salud en sectores pobres y carentes de servicios sanitarios básicos con altas tasas de prevalencia (Romero-Sandoval et al., 2017; Cepon-Robins et al., 2014).

1.4 **Trematodosis transmitidas por alimentos**

Es el mayor grupo dentro de las NTDs. Se estima que cerca de 40 millones de personas están infectadas y más de 750 millones están en riesgo de infectarse (más del 10% de la población mundial), y que habitan principalmente en el sudeste asiático (Hotez et al., 2008; Keiser & Utzinger, 2005).

Se estima que la carga global de estas enfermedades es de 665.352 años de vida perdidos, debidos a la discapacidad que producen (Fürst et al., 2012). Además, en 1994, la Agencia Internacional de Investigación del Cáncer (IARC; del inglés International Agency for Research on Cancer) clasificó a *O. viverrini* y *C. sinensis* como agentes carcinogénicos (Working Group of the International Agency for Research on Cancer, 1994).

Estas enfermedades causadas por parásitos que pertenecen al *Phylum* Platyhelminthes, clase Trematoda, subclase Digenea, son clasificadas de acuerdo a la localización anatómica de los parásitos adultos en el organismo del hospedador definitivo. Más de 80 especies han sido reportadas infectando a humanos (Chai, 2007; Blair et al., 2007; Mas-Coma et al., 2007; Fürst et al., 2012). Se localizan principalmente en pulmones, vías biliares y tubo digestivo (Keiser & Utzinger, 2007) (Tabla 1), y mantienen complejos ciclos de vida en la naturaleza, los cuales han sido bien detallados en la literatura (Keiser et al., 2009).

Tabla 1. Trematodos transmitidos por alimentos de importancia médica y de Salud Pública.

Órganos afectados	Género	Especie	Fuente infección humana	Hospedador natural final de la infección
Hígado y vías biliares	*Clonorchis*	*C. sinensis*	Peces	Perros y otros carnívoros que consumen peces.
	Opisthorchis	*O. viverrini* *O. felineus*	Peces	Gatos y otros carnívoros que consumen peces.
	*Amphimerus**	*Amphimerus* spp.	Peces	Gatos, perros, aves, ratones, tortugas, raposas, delfines.
Pulmones	*Paragonimus*	*Paragonimus* spp.	Cangrejos de río	Gatos, perros y carnívoros que comen crustáceos.
	Fasciola	*F. hepatica* *F. gigantica*	Vegetales acuáticos	Ovejas, ganado y otros herbívoros
Intestino	*Haplorchis*	*H. taichui,* *H. pumilio*	Peces	Pájaros y otros mamíferos que consumen peces.
	Metagonimus	*M. yokogawa*	Peces	

* Parásito reportado por nuestro grupo de investigaciones.

Fuente: WHO 2011; Sripa et al., 2010; Calvopiña et al., 2012.

A pesar de que han sido descritos hace cientos de años (Mas-Coma et al., 2005), aún existen dudas respecto a su taxonomía, ya que nuevas especies continúan siendo identificadas y descritas (Chai, 2007; Blair et al., 2007).

Más de 18 millones de personas en el mundo se ven afectadas por la infección de trematodos hepáticos pertenecientes a la familia Opisthorchiidae (Chai et al., 2009; WHO, 1995) y más de 500 millones están en riesgo de ser infectados (dos Santos & Howgate, 2011). La tabla 2, muestra las principales especies de trematodos hepáticos transmitidas por alimentos de importancia en Salud Pública, especificando la fuente de transmisión y la zona endémica donde se producen.

Tabla 2. Principales especies de trematodos hepáticos transmitidos por alimentos de importancia en Salud Pública.

Familia	Género	Especie	Fuente infección humana	Zona endémica
Opisthorchiidae	*Clonorchis*	*C. sinensis*	Pescado	Asia Oriental.
	Opisthorchis	*O. viverrini*	Pescado	Asia Oriental.
		O. felineus	Pescado	Asia Central y Este de Europa.
	Amphimerus	*Amphimerus* spp.	Pescado	Américas y Asia.
Fasciolidae	*Fasciola*	*F. hepatica*	Plantas	Europa, Asia y
		F. gigantica	Plantas	América.

Las trematodosis zoonóticas transmitidas a través del consumo de peces crudos o insuficientemente cocinados son denominadas ictiozoonosis (Tabla 3), las cuales han generado un gran problema de Salud Pública en el mundo (WHO, 1995; dos Santos & Wowgate, 2011). Entre los trematodos que afectan al hombre, más de 50 son transmitidos por el consumo de peces (Furst & Uzinger, 2012; Hung et al., 2013). Entre ellas, encontramos a la amphimeriosis, producida por *Amphimerus* spp., que es conocida desde hace más de 100 años como una infección de reptiles, aves y ciertos mamíferos (Calvopiña et al., 2011). Al parecer, este parásito fue descrito anteriormente infectando a humanos en Ecuador bajo la denominación de *Opisthorchis guayaquilensis* (Rodriguez et al., 1948, Moreira et al., 2008).

Las especies de mayor importancia para la salud humana son las ictiozoonosis que afectan al hígado, producidas por parásitos de la familia Opisthorchiidae, los cuales parasitan los conductos biliares pequeños del hospedador humano o animal. Tienen una amplia distribución a nivel mundial, presentando alta prevalencia especialmente en países asiáticos, causando elevada morbilidad (Chai et al., 2005; Keiser & Utzinger, 2009; dos Santos et al., 2011; Mas-Coma & Bergues, 1997). Por ejemplo, en Taiwán, *C. sinensis* tiene tasas de prevalencia de hasta un 57%. Se estima que una región con una prevalencia mayor al 20% debe ser considerada como área hiperendémica y donde el tratamiento masivo con praziquantel estaría justificado (Chen, 1991; Rim, 1986). Estas infecciones son reconocidas por causar enfermedad grave en ciertas zonas geográficas del mundo. Colangitis, coledocolitiasis, pancreatitis y colangiocarcinoma se asocian a un patrón de infección crónica (Chai et al., 2009; Mas-Coma & Bergues, 1997).

Tabla 3. Ictiozoonosis reportadas en humanos y animales a nivel mundial.

Familia	Género	Especie	Zona endémica
Localización hepática y vías biliares			
Opisthorchiidae	*Clonorchis*	*C. sinensis*	Asia Oriental.
	Opisthorchis	*O. viverrini*	Asia Oriental.
		O. felineus	Asia Central y Este de Europa.
		Opisthorchis tenuicollis	Asia y Europa.
	Amphimerus	*Amphimerus* spp.	Américas, Asia.
		Metorchis albidus	Europa, Asia.
		Metorchis bilis	Europa.
		Metorchis conjunctus	Norte América
		Metorchis orientalis	Asia Oriental.
	Pseudamphistomum	*Pseudamphistomum* spp.	África, Europa y Norte América.
Localización intestinal			
Nanophyetidae	*Nanophyetus*	*Nanophyetus salmincola*	Rusia, Estados Unidos América.
Echinostomatidae	*Echinochasmus*	*Echinochasmus* spp.	Asia y África.
	Echinoparyphium	*E. paraulum*	Asia, Europa,
	Echinostoma	*Echinostoma* spp.	Nueva Zelandia,
	Episthmium	*E. caninum*	Brasil
Heterophyidae,	*Appophalus*	*Appophalus donicus*	Europa, Canadá.
	Ascocotyle	*Ascocotyle* spp.	Asia, África,
	Centrocestus	*Centrocestus* spp.	América, Europa.
	Cryptocotyle	*Cryptocotyle lingua*	Asia, Europa, EUA
	Haplorchis	*Haplorchis* spp.	Asia, África,
	Heterophyes	*Heterophyes* spp.	América.
	Heterophyopsis	*Heterophyopsis continua*	Asia.
	Isthimiophora	*Isthimiophora melis*	Asia, EUA, Europa
	Metagonimus	*Metagonimus* spp.	Asia
	Phagicola	*Phagicola sp.*	Las Américas
	Procerovum	*Procerovum* spp.	Asia, África.
	Pygidiopsis	*Pygidiopsis summa*	Asia Oriental.
	Stellantchasmus	*Stellantchasmus* spp.	Asia Oriental.
	Stictodora	*Stictodora* spp.	Corea y Japón.

Fuente: Hung et al., 2013.

Varias son las razones para el incremento de estas ictiozoonosis: el desarrollo de nuevas y mejores técnicas diagnósticas, el incremento de consumo de pescado mal cocinado en las poblaciones endémicas en forma de sashimi, sushi, ceviche, encurtido; el incremento en viajes y comercio de pescado de cultivo en criaderos tanto para consumo local como para exportación, entre éstos la tilapia y truchas, el desarrollo de la acuacultura y el descubrimiento de estas parasitosis en zonas donde antes no se había documentado la transmisión (dos Santos & Wowgate, 2011; Calvopiña et al., 2011; Hung et al., 2013).

Estas enfermedades de origen alimentario se desarrollan en un ambiente eco-epidemiológico específico en el que confluye un pobre saneamiento e higiene, hábitos culturales de consumo de alimentos, presencia de reservorios domésticos y silvestres, variedad de hospedadores intermediarios, pobreza y un diagnóstico tardío, ya que muchas infecciones pasan desapercibidas. La pobreza como expresión social de la falta de escolaridad, baja calidad de la vivienda, falta de acceso a agua segura y pobre saneamiento ambiental es uno de los determinantes sociales claves para el mantenimiento y el aumento de estas enfermedades que producen discapacidad, una importante morbi-mortalidad a largo plazo y disminución de la productividad, lo cual genera un círculo vicioso de pobreza-enfermedad-pobreza (WHO, 2010; Hotez, 2007; Liese et al., 2010; Hotez et al., 2010).

Otros determinantes sociales de la salud son por ejemplo, el papel social asignado a las mujeres y niños como agentes de recolección de agua y juego dentro de ríos, lo cual les expone más a enfermedades vectoriales y consumo de alimentos poco o mal preparados. Esto representa una dinámica de trasmisión de la enfermedad específica que requiere intervenciones focalizadas como acceso a agua segura, educación y mejora en el saneamiento (Guernier et al., 2004; Rathgeber et al., 1993). Las condiciones de trabajo dedicados a agricultura y pesca de subsistencia determinan un mayor riesgo de contraer las NTDs (Coutinho et al., 1997). También el bajo nivel de escolarización les impide conocer las formas de evitar o disminuir el riesgo de exposición, comparado con poblaciones con mayores niveles de educación (Conteh et al., 2010, Vecchiato, 1997; Ho, 2004; Boelaert et al., 2010).

El saneamiento inadecuado influye en su transmisión, ya que la falta de una adecuada eliminación de excretas aumenta el riesgo de infección de los hospedadores intermediarios. Así, la mejora del abastecimiento de agua y construcción de letrinas adecuadas, son medidas imprescindibles para mejorar el saneamiento y reducir la transmisión de estas enfermedades (Relman & Choffnes, 2011; Aagaard-Hansen, 2010; Fuller et al., 2016).

El control de la exposición humana a los hospedadores intermediarios, como son los pescados de agua dulce, resulta ser un componente crítico en la prevención ante la falta de vacunas y la imposibilidad de su eliminación dada la presencia de otros reservorios domésticos y silvestres (WHO, 2013; Karesh et al., 2012).

Entre las trematodosis transmitidas por alimentos en el Ecuador, destacan la paragonimosis pulmonar, comúnmente subdiagnosticada y transmitida por la ingestión de cangrejos dulceacuícolas insuficientemente cocinados (Calvopiña et al., 2014); también la fasciolosis, la cual se contrae por la ingesta de plantas acuáticas infectadas con metacercarias que son consumidas crudas en ensaladas. Esta parasitosis es endémica en ciertas comunidades alto andinas con presencia de ambientes ecológicos propicios para su transmisión (Trueba et al., 2000).

Los esfuerzos para la prevención y el control de estas enfermedades deben iniciarse con la identificación de la carga de infección, los hospedadores intermediarios involucrados en la transmisión y los factores de riesgo asociados. De esta manera se podrán llevar a cabo campañas de prevención mediante educación sanitaria a la población para evitar el consumo de hospedadores intermediarios o promoviendo su consumo adecuado. Adicionalmente, otros métodos para promover un control integrado se basan en mejorar el saneamiento e higiene en la población (Relman & Choffnes, 2011; Rollinson et al., 2013; Mazigo et al., 2012).

La OMS recomienda 5 estrategias para el control de estas enfermedades: *(i)* quimioterapia preventiva, *(ii)* manejo de la enfermedad y acceso a la atención especializada, *(iii)* control de hospedadores intermediarios, *(iv)* atención a la salud pública veterinaria en su relación con la salud humana y, *(v)* provisión de agua segura, saneamiento e higiene (WHO, 2012).

Dentro de la subclase de trematodos digénidos, está la familia Opisthorchiidae. Los más importantes desde el punto de vista de Salud Pública son *Clonorchis sinensis*, *Opisthorchis viverrini* y *O. felineus* que infectan al hombre y otros reservorios por comer pescado de agua dulce en platos típicos como sushi y sashimi y que son endémicos en el sudeste del continente asiático (Sripa et al., 2007). Dentro de esta familia, en los últimos años se ha reportado la infección causada por *Amphimerus* spp., trematodo hepático descubierto en poblaciones indígenas del noreste de la costa Pacífica ecuatoriana (Calvopiña et al., 2011) y que es objeto de estudio de la presente tesis doctoral.

1.5 *Amphimerus* spp. y amphimeriosis

1.5.1 Definición

El parásito trematodo hepático *Amphimerus* spp. debe su nombre a los vocablos latinos *Amphi* que significa "en ambos sitios" y *merus* "posterior", refiriéndose a la disposición de sus glándulas vitelinas en el estadio adulto, las cuales están divididas en dos grupos, un grupo de dos anteriores y un grupo de dos posteriores a nivel del ovario.

La amphimeriosis es la enfermedad zoonótica causada por *Amphimerus* spp*.* que se transmite al ingerir pescados de río crudos o escasamente cocinados.

1.5.2 Breve reseña histórica

Dentro del género *Amphimerus*, el primer trematodo de vías biliares estudiado fue *Amphimerus pseudofelineus*, descrito originalmente por Ward en 1901 en un gato de Nebraska, Estados Unidos de América. Posteriormente, Barker en 1911 lo clasificó dentro del género *Amphimerus* diferenciándolo de aquellos pertenecientes a la familia Opisthorchiidae como *Clonorchis sinensis* y *Opisthorchis viverrini.* A partir de entonces, *A. pseuodofelineus* ha sido descrito ampliamente en las Américas infectando también a gatos en los estados de Illinois y Michigan. Además, se ha estudiado el parásito a través de infecciones experimentales en Manitoba, Canadá.

En 1948, Rodríguez y colaboradores descubren un trematodo hepático infectando a humanos en Ecuador, denominándolo *Opisthorchis guayaquilensis* (Rodriguez et al., 1948). Yamaguti en 1971, indica que este parásito previamente reportado en Ecuador, podría tratarse de *Amphimerus* spp. (Yamaguti, 1971). Así, es referido con este nombre en otras publicaciones posteriores (de Moraes N et al., 1998). No obstante, son necesarios estudios moleculares para identificar con exactitud este trematodo de vías biliares.

1.5.3 Taxonomía

La taxonomía básica de los trematodos transmitidos por alimentos se muestra en la figura 2, enfatizando a aquellos que infectan al hombre. Hemos incluido a *Amphimerus* spp. recientemente descubierto por nuestro grupo de investigación (Keiser & Utzinger, 2009; Calvopiña et al., 2011).

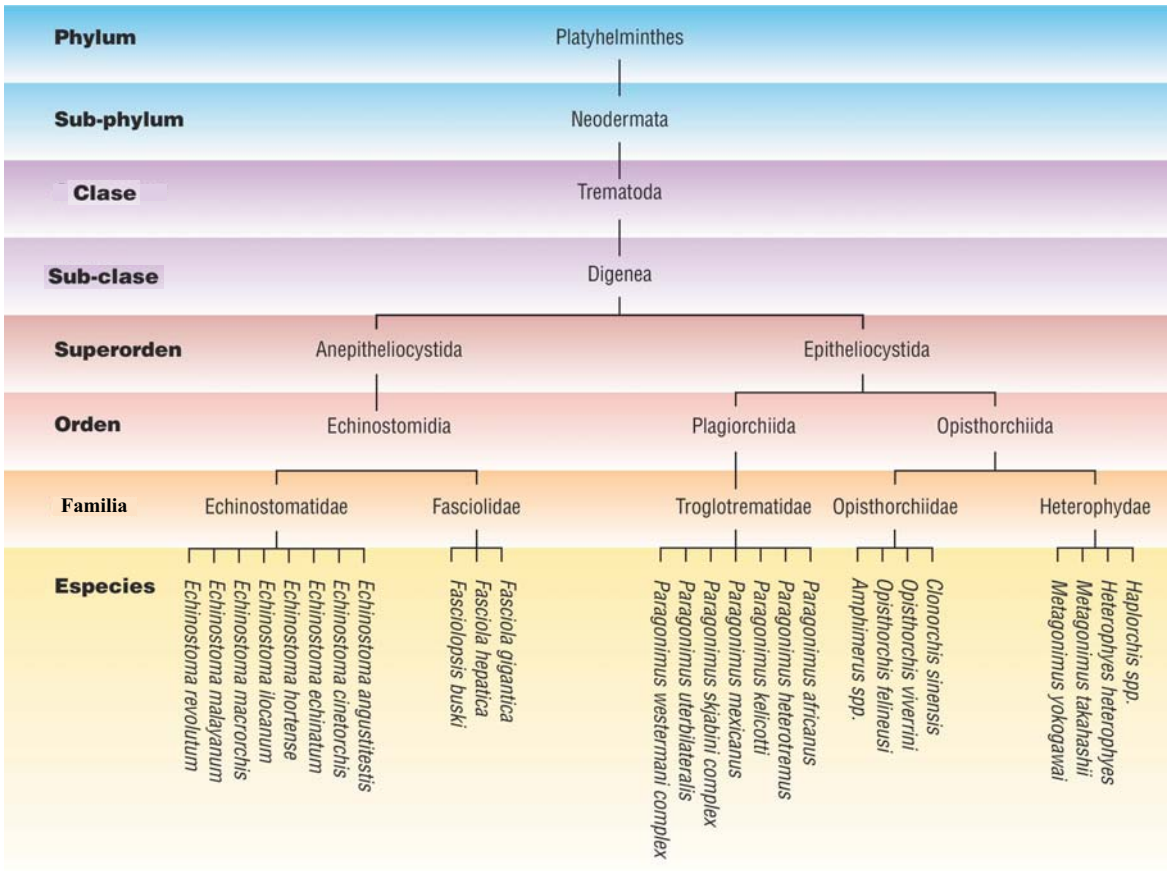


Figura 2. Clasificación de los principales trematodos transmitidos por alimentos.

El género *Amphimerus* (Barker, 1911) pertenece al phylum *Platyhelminthes*, clase Trematoda, subclase *Digenea*, familia *Opisthorchiidae* (Calvopiña et al., 2011). Está estrechamente relacionado con los trematodos hepáticos de la familia Opisthorchiidae*: Opisthorchis* spp. y *Clonorchis sinensis*. Infecta a mamíferos del continente Americano incluyendo Canadá, Estados Unidos, México, Costa Rica, Panamá, Venezuela, Colombia, Ecuador, Brasil y Perú; también en el sur de Asia, Corea del Sur, India y Filipinas (Bowman, 2002; Yamaguti, 1971; Miyazaki et al., 1978; Rivillas et al., 2004; de Moraes Neto et al., 1998; Artigas et al., 1962; Thatcher, 1970; Calvopiña et al., 2011, Eom et al., 1984). No se ha reportado su presencia en otros continentes como África, Oceanía, Asia o Europa. Actualmente, han sido descritas las siguientes especies: *A. lancea* (Diesing, 1850), *A. speciosus* (Stiles & Hassall, 1896), *A. interruptus* (Braun, 1901), *A. pseudofelineus* (Ward, 1901), *A. noverca,* en India (Braun, 1902), *A. ovalis* (Barker, 1911) que es la especie tipo*, A. anatis* (Yamaguti, 1933), *A. elongatus* (Gower, 1938), *A. pricei* (Foster, 1939). *A. caudalitestis* (Caballero, Grocott & Zerecero, 1953), *A. neotropicalis* (Caballero, Geis & Caballero, 1963), *A. parciovatus* (Franco, 1967) y *A. bragai* N.sp. (de

Moraes Neto A et al., 1988). De éstos, *A. ovalis* es un trematodo de reptiles; *A. speciosus, A. interruptus, A. anatis* y *A. elongatus,* son parásitos de aves; las demás son parásitos de mamíferos (Franco, 1967).

1.5.4 Morfología

Amphimerus spp. es un trematodo hepático hermafrodita. Los parásitos adultos habitan en los conductos biliares pequeños y medianos. Son de morfología aplanada y alargados en forma de hoja, presentan una coloración rojo- rosada y miden entre 8-13,6 mm de largo por 0,5-1,1 mm de ancho. Pasados unos minutos en solución salina los gusanos se enrollan en forma de "S" y se vuelven transparentes o blanquecinos. En su estructura destacan las glándulas vitelinas distribuidas en cuatro grupos, 2 anteriores y 2 posteriores; la ventosa ventral es mayor que la oral y los testículos son redondeados o ligeramente lobulados (Calvopiña et al., 2011). En contraste, las glándulas vitelinas en *Clonorchis* y *Opisthorchis* existen solo en la zona frontal a los testículos (Figura 3). Adicionalmente, *Clonorchis* tiene dos testículos grandes altamente ramificados y en *Opisthorchis* son siempre lobulados (Yamaguti, 197; Bowman, 2002).

Los huevos presentan un opérculo en su parte anterior y una espina inferoposterior. Tienen un tamaño de 28-33 µm x 12-15 µm. A la observación por microscopía electrónica, la superficie de los huevos de *Amphimerus* spp. muestra diferencias con los huevos de otros trematodos de la familia Oposthorchiidae. Así, mientras es rugosa e irregular en forma de parches en *Amphimerus* spp., es fina, regular y reticulada en *C. sinensis* (Figura 4).

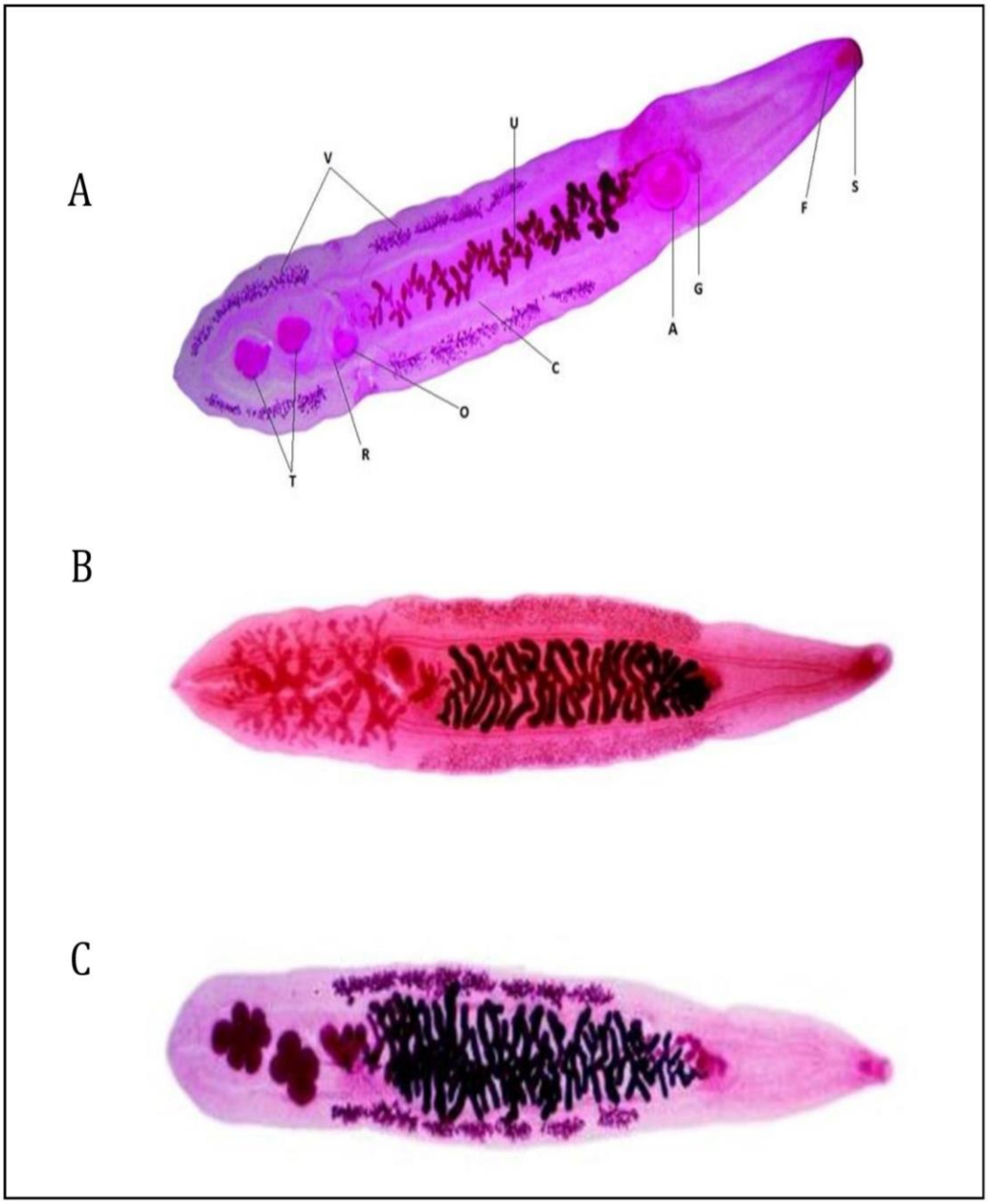


Figura 3. Características morfológicas de *Amphimerus* spp, *Clonorchis sinensis* y *Opisthorchis viverrini*. **A**. *Amphimerus* spp. adulto de Ecuador (corte ventral). Ventosa ventral (A) es mayor que la Ventosa oral (S). Poro genital (G) se abre delante de la ventosa ventral; Glándulas vitelinas (V) están divididas en dos partes; los Testículos (T) son redondeados. Estas son las diferencias comparadas con los parásitos trematodos asiáticos *O. viverrini* y *C. sinensis*. R: Receptáculo seminal, O: Ovario, U: Útero, C: Conducto vitelino, y; F: Faringe. Comparado con **B**. *Clonorchis sinensis*, y; **C**. *Opisthorchis viverrini* (Sripa et al., 2007).

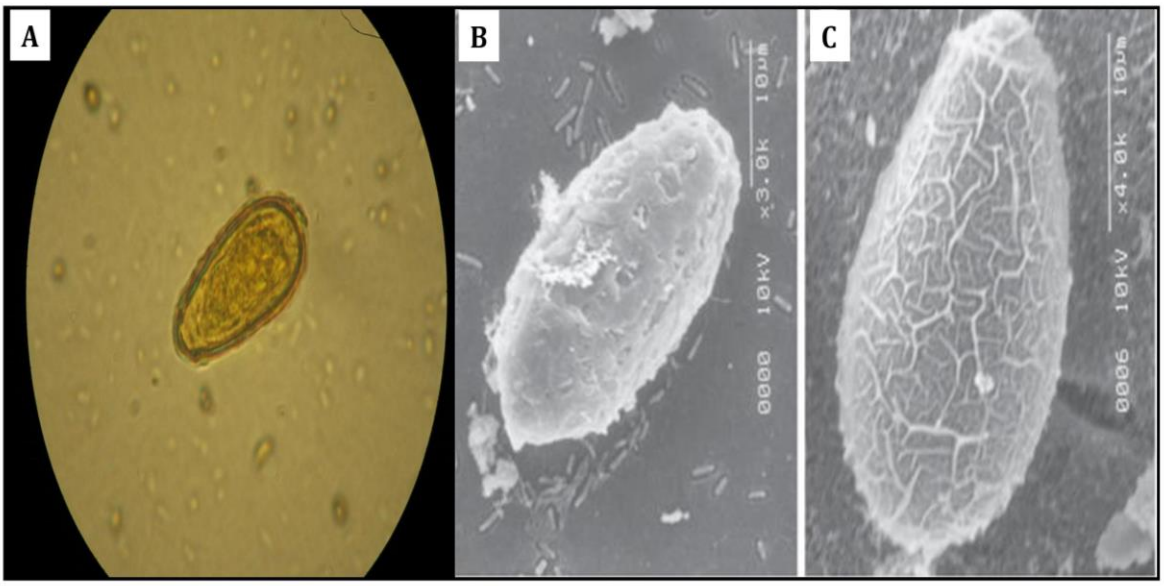


Figura 4. Imágenes de microscopía óptica y electrónica de huevo de *Amphimerus* spp. y *Clonorchis sinensis.* (**A**) Huevo de *Amphimerus* spp. ecuatoriano obtenido de persona a microscopía óptica (X 3) y (**B**) a microscopía electrónica. **(C)** Huevo de *C. sinensis* a microscopía electrónica. A pesar del tamaño similar, las diferencias en la superficie permiten diferenciar los dos géneros (Calvopiña et al., 2011).

1.5.5 Ciclo biológico

Conocemos muy poco acerca del ciclo biológico de *Amphimerus* spp. Sin embargo, nuestras primeras investigaciones evidencian que este parásito produce infección en humanos y animales domésticos a través de la ingestión de peces crudos o insuficientemente cocinados. Al igual que en otros trematodos de la familia Opisthorchiidae, los peces son portadores de metacercarias activas, las cuales una vez ingeridas se separan de la carne del pescado en el estómago por la acción del jugo gástrico, avanzando hasta el duodeno, donde son liberadas por acción combinada de proteasas como tripsina y cisteína. Posteriormente, los trematodos liberados atraviesan la ampolla de Vater y alcanzan las vías biliares intrahepáticas, sitio en el cual se desarrolla el parásito adulto, pudiendo vivir hasta 30 años y produciendo aproximadamente 4.000 huevos por día. (Attwood, 1978; Kim et al., 2009). La maduración a parásito adulto se produciría aproximadamente en dos meses, como en el caso de *O. viverrini* y *C. sinensis.* Los humanos, reptiles y ciertos mamíferos como perros y gatos que ingieren peces de río pueden servir como hospedadores definitivos (Taylor et al., 2001).

Una vez iniciada la reproducción sexual, el parásito adulto produce huevos embrionados los cuales pasan a las vías biliares y son eliminados a través de las heces

en las orillas de los ríos o lagunas. Los huevos son ingeridos por un caracol, que sirve de primer hospedador intermediario. Cada huevo libera un miracidio, el cual atraviesa varias etapas de desarrollo intramolusco (esporoquistes, redias y cercarias). Las cercarias liberadas por el caracol tras un breve período de tiempo en el agua, penetran en la carne de un pez de agua dulce que actúa como su segundo hospedador intermediario (Figura 5).

Existen más de 100 especies de caracoles que pueden servir de hospedadores intermediarios primarios de diferentes trematodos. Hasta el momento, desconocemos que género y especie de caracol sirve como primer hospedador intermediario de *Amphimerus* spp. A través de la técnica de PCR-RFLP, se han logrado identificar metacercarias de *Amphimerus* spp. en cuatro especies de peces de agua dulce: *Rhoadsia altipinna* (familia Characidae), *Bryconamericus bucay*, *Andinoacara rivulatus* (familia Cichlidae) y *Lebiasina aureoguttata* (familia Piabucinae; Fowler, 1911) (Figura 6). Las tasas de infección natural fueron del 80%, 10%, 18% y 1% respectivamente. La identificación de estas 4 especies de peces transmisores de *Amphimerus* spp. que incluyen al menos 3 familias taxonómicas, sugiere que este trematodo tiene varios peces como hospedadores intermediarios secundarios (Calvopiña M, comunicación personal).

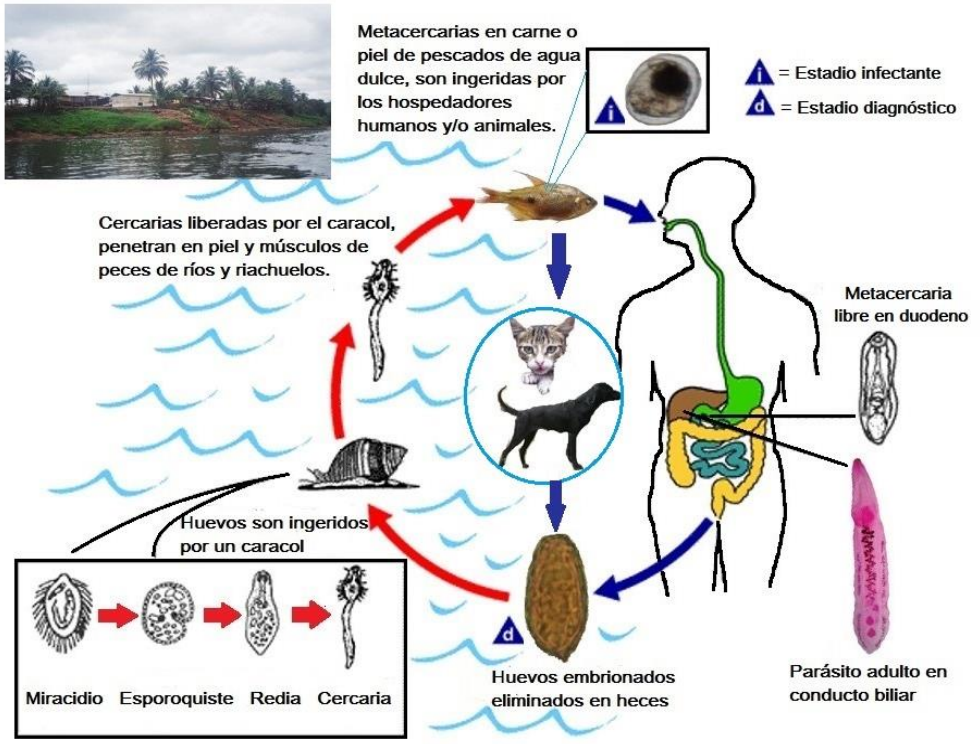


Figura 5. Ciclo biológico de *Amphimerus* spp.

(Adaptado de http://www.dpd.cdc.gov/DPDx/HTML/Opisthorchiasis.htm).

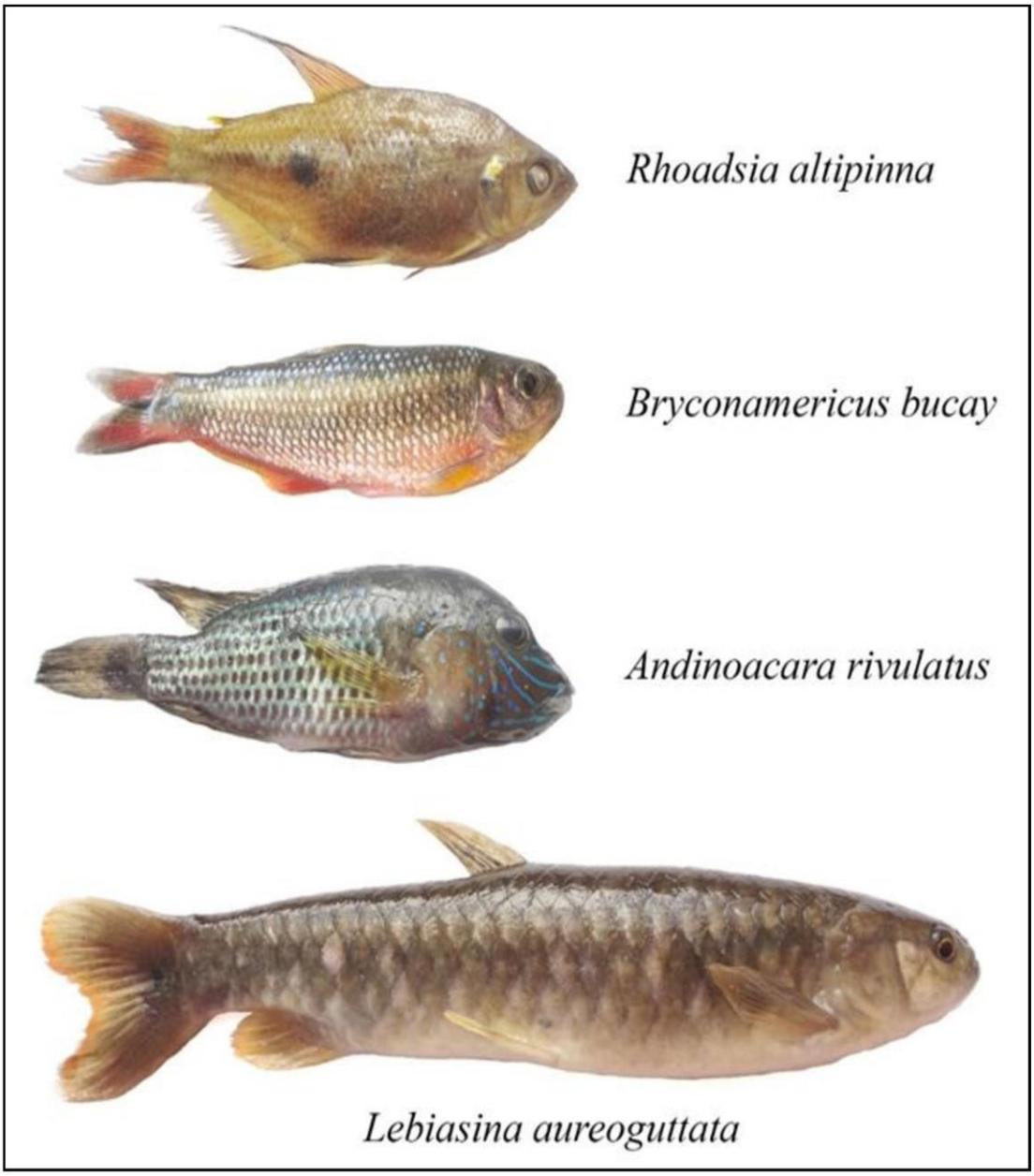


Figura 6. Peces de agua dulce involucrados en la transmisión de *Amphimerus* spp. en Ecuador.

(Fotografía cortesía de Dr. Daniel Romero)

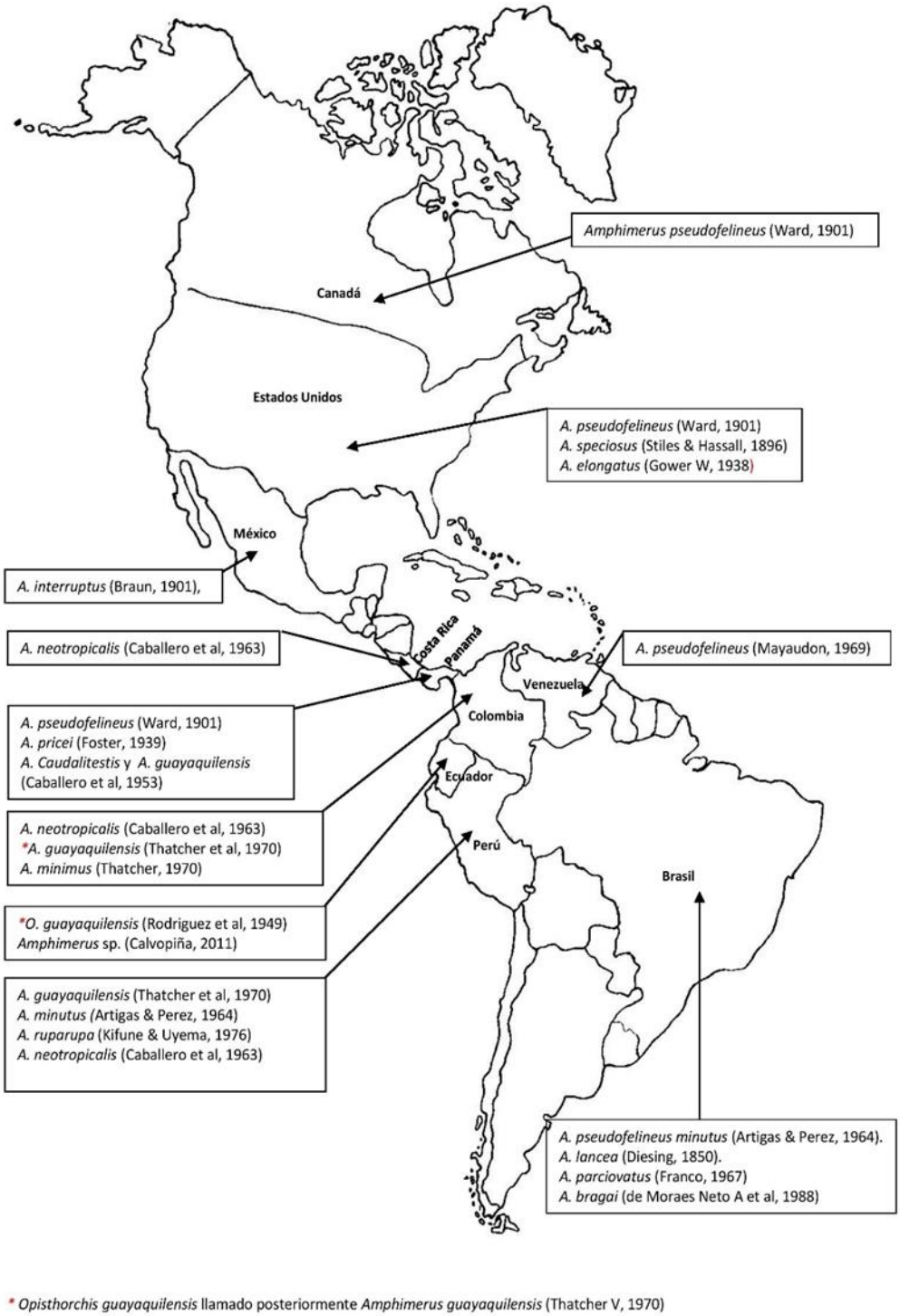
1.5.6 Epidemiología

En la actualidad, la infección en humanos por *Amphimerus* spp. ha sido reportada solamente en Ecuador (Calvopiña et al., 2011). Sin embargo, se han identificado infecciones por el parásito en otros hospedadores vertebrados en diferentes países del continente americano con el consiguiente riesgo de infección para la población humana (Thatcher, 1970; Miyasaki et al., 1976; de Moraes et al., 1998). Así, existen informes en Colombia y Perú donde se han identificado huevos pertenecientes a la familia *Opisthorchiidae* en heces humanas semejantes a *Amphimerus* spp. (Restrepo M, 1962; Beltran M. & Naquira C, 1999). Estas evidencias demuestran la importancia del estudio de esta parasitosis en humanos en países vecinos a Ecuador.

Respecto a las especies de *Amphimerus* spp. identificadas en hospedadores no humanos, en la figura 7 se muestra un mapa del continente americano con catorce especies de parásitos descritas hasta la fecha. Esta información se completa con los datos referidos en la tabla 4 en la que se detalla los hospedadores en los que se aislaron las diferentes especies del parásito.

Al igual que en otras trematodosis de origen alimentario, la prevalencia e intensidad de la amphimeriosis está determinada por la cultura profundamente arraigada entre determinadas poblaciones de ingerir pescado de rio crudo o insuficientemente cocinado. En zonas endémicas para *C. sinensis* y *Opisthorchis* spp. existe la creencia de que el consumo de pescado crudo es altamente nutritivo (Phan et al. 2011; Qian et al., 2013).

Entre los determinantes ambientales y ecológicos que afectarían la endemicidad de esta parasitosis, sería de gran importancia conocer las especies de hospedadores intermediarios primarios (caracoles terrestres) y secundarios (peces de agua dulce). A día de hoy no se conocen los caracoles transmisores de *Amphimerus* spp., aunque en el caso de trematodos afines como *C. sinensis,* las especies identificadas han sido *Parafossarulus striatulus, Bithynia fuchsiana, Alocinma longicornis* y *Melanoides tuberculata* (Lun et al., 2005; WHO, 1995). En cuanto al segundo hospedador intermediario, estudios preliminares realizados por nuestro grupo dan cuenta de que la mayoría de especies de peces infectadados con metacercarias de *Amphimerus* spp. corresponden al género *Rohadsia altipinna,* pertenecientes a la familia Characidae (datos no publicados). Animales domésticos como perros y gatos, sirven también como hospedadores definitivos de *Amphimerus* spp., actuando tambien como reservorios, pudiendo mantener el ciclo de vida del parásito sin la participación del hombre.



* *Opisthorchis guayaquilensis* llamado posteriormente *Amphimerus guayaquilensis* (Thatcher V, 1970)

Figura 7. Distribución geográfica de *Amphimerus* spp. en las Américas.

Tabla 4. Distribución de *Amphimerus* spp. y sus hospedadores definitivos en las Américas.

Parásito	Hospedador	País (localidad)	Registro
A. pseudofelineus	No reportado *Felis felis (Catus) domesticus* *Felis felis (Catus) domesticus*	Canadá (Lago Minatoba) Venezuela (Maracay-Aragua) Estados Unidos de Norteamérica	Ward, 1901 Mayaudon, 1969
A. speciosus	No reportado	Estados Unidos de Norteamérica	Stiles & Hassall, 1896
A. pseudofelineus minutus	*Dildelphis marsupialis.*	Brasil (Amazonía)	Artigas & Pérez, 1964
A. lancea	*Sotalia tucuxi* y *Nectomys squamipes*	Brasil (Amazonía, Goiás)	Diesing, 1850
A. parciovatus	*Dildelphis marsupialis marsupialis L.*	Brasil (Belém do Pará)	Franco, 1967
A. bragai	*Nectomys squamipes*	Brasil (Minas Gerais)	de Moraes Neto A et al., 1988
A. minutus	*Dildelphis marsupialis marsupialis*	Perú (Huánuco)	Artigas & Perez, 1964
A. ruparupa	*Philander opossum*	Perú (Huánuco y Loreto)	Kifune & Uyema, 1976
O. guayaquilensis X *A. guayaquilensis*	*Cannis familiaris* *Philander opossum L. &* *Dildelphis marsupialis L.* *Dildelphis marsupialis &* *Felis felis (Catus) domesticus*	Ecuador (P.P. Gómez) Colombia (Buenaventura, Valle y Antioquia) Panamá (Colón)	Rodríguez et al., 1949 Thatcher V, 1970 Caballero et al., 1953
A. neotropicalis	*Philander fpossum L. &* *Dildelphis marsupialis L.* *Philander opossum* *Dildelphis peruguayensis*	Colombia (Buga y Villa Carmelo, Valle) Costa Rica Perú	Thatcher V, 1970 Caballero et al., 1963 Miyazaki I, 1991
A. minimus	*Philander opossum L.*	Colombia (Buga, Valle)	Thatcher, 1970
A. pricei	*Caluromys derbianus*	Panamá	Foster, 1939
A. caudalitestis	*Chironectes minimus*	Panamá	Caballero et al., 1953
A. interruptus	No reportada	México	Braun, 1901
A. elongatus	*Phalacrocorax auritus, birds.* *Ducks, swans*	Estados Unidos (Louisiana y Michigan)	Pense & Childs, 1972 Gower W, 1938
Amphimerus spp.	*Homo sapiens sapiens,* *Cannis familiaris* y *Felis felis (Catus domesticus)*	Ecuador (Río Cayapas-Esmeraldas)	Calvopiña et al., 2011

1.5.7 Mecanismos patogénicos

Los mecanismos de agresión, defensa y evasión inmunológica desencadenados en la amphimeriosis son desconocidos. Sin embargo, estos mecanismos están bien descritos en otros trematodos de la familia Opisthorchiidae como *C. sinensis* y *O. viverrini*, los cuales son responsables de producir infecciones de larga duración, e incluso desencadenar carcinoma de vías biliares o colangiocarcinoma (CCA) (Choi et. al., 2004; Sripa et al., 2007; Sripa et al., 2012).

Basados en estudios de estos trematodos, mayoritariamente realizados en modelos experimentales, describiremos a continuación los diferentes mecanismos patogénicos conocidos hasta el momento.

Mecanismos de agresión

Se pueden agrupar en tres acciones principales: *(i)* daño mecánico, *(ii)* efecto tóxico, y *(iii)* proceso inflamatorio.

Es evidente que *Amphimerus* spp. debería ocasionar cambios en los conductos biliares originando **procesos obstructivos y de alteración de la mucosa**. Daños similares se han documentado en la infección por *C. sinensis* durante el proceso de alimentación a través de su ventosa oral (Sripa et al., 2010).

Para sobrevivir largos períodos en entornos hostiles como el fluido biliar, los trematodos hepáticos liberan activamente **productos de excreción/secreción (ES)** a través del tegumento y del poro excretor, algunos de los cuales son altamente inmunogénicos (Sripa et al., 2000; Mulvenna et al., 2010). Además, pueden ser tóxicos o interactuar con el epitelio biliar para estimular la inflamación, promover la proliferación y suprimir la apoptosis (Kim et al., 2008; 2009). Varios estudios demuestran que los productos de ES del parásito adulto *C. sinensis* provocan cambios en el perfil de expresión del transcriptoma, proteoma y micro ARN en células humanas (HuCCT1) con CCA y en hígados de ratones (Pak et al., 2009; 2014). Además, estos productos pueden producir hiperplasia de células biliares normales a células adenomatosas con posterior transformación a CCA, debido a la alteración transcripcional de genes diana carcinogénicos, tales como el gen Mcm7, a través de modificaciones de histonas (Sripa et al., 2010). Con los recientes avances en la caracterización del transcriptoma (Laha et al., 2007; Young et al., 2010) y del proteoma de productos de ES de *O. viverrini* y *C. sinensis* (Mulvenna et al., 2010; Pak et al., 2009), se han identificado varias moléculas del parásito con actividad carcinogénica (Daorueang et al., 2012; Smout et al., 2009). Entre ellas destaca una proteína con gran similitud al factor de crecimiento en mamíferos, denominada granulina (Ov-GNR-1).

Esta proteína es el único factor de crecimiento derivado de helmintos reportado hasta la fecha que puede causar la proliferación de células de mamíferos (Smout et al., 2009).

Una pro-granulina humana (PGRN), se asocia con muchos cánceres agresivos y se sobre expresa en muchos tumores humanos (Bateman & Bennett; 1998). Se ha visto además que la PGRN estimula la angiogénesis, suprime la apoptosis, promueve la invasión tumoral y apoya la expansión del tumor en un entorno intersticial desfavorable (Ong et al., 2003; Zanocco-Marani et al., 1999). De hecho, la expresión de PGRN impulsada por IL-6 da como resultado un mayor crecimiento de CCA (Frampton et al., 2012). Sería por tanto de gran interés conocer el genoma, transcriptoma y proteoma de *Amphimerus* spp. con la finalidad de identificar moléculas clave en la relación parásito-hospedador y en la posible dilucidación de un efecto carcinogénico.

Los Toll-like receptors (TLRs) son precursores muy potentes de la **respuesta inflamatoria** y permiten el reconocimiento de patógenos incluso en la fase inicial de la infección (Kawai & Akira; 2011). Sin embargo, en la inflamación prolongada existe una producción excesiva de citocinas inflamatorias mediada por los TLRs, lo cual podría ser perjudicial por causar toxicidad en el hospedador con el consiguiente daño tisular (Sripa et al., 2012). En infecciones experimentales con *C. sinensis*, los TLR2 y TLR4 fueron altamente expresados en ratones, lo que indica una probable participación de estos TLRs en la estimulación de la respuesta inmune innata (Yan et al., 2015). Además, para estudiar las primeras etapas de la inmunopatología en el tracto biliar de las personas infectadas con *O. viverrini*, se estimuló una línea celular de colangiocitos humana normal (H69) con productos de ES del parásito, induciendo una mayor expresión de TLR4 (Ninlawan et al., 2010). Por otro lado, diversos estudios han demostrado niveles altos de IL-6 específica contra los productos de ES del parásito en pacientes con fibrosis periductal avanzada (Sripa et al., 2009). Esta citocina también podría estar implicada en otras anomalías hepatobiliares incluido el CCA (Sripa et al., 2012). Estos trabajos indican nuevamente la importancia de estudiar la respuesta inflamatoria desencadenada en la infección por *Amphimerus* spp. Una primera limitación se debe a la inexistencia en la actualidad de modelos experimentales de infección con este trematodo.

Mecanismos de defensa

Es bien conocido que la generación de respuestas inmunológicas Th1-dependientes, están no solamente asociadas con la eliminación del parásito, sino también, con la inducción de lesiones graves en el propio hospedador. Por el contrario, la activación de la vía tipo Th2 conduce al desarrollo de respuestas inmunológicas beneficiosas para el parásito, facilitando la progresión de la enfermedad (Liu & Ding; 2016). En este sentido, la infección crónica producida por *C. sinensis*, se asocia predominantemente a

respuestas tipo Th2, induciendo la supresión de citocinas involucradas en la respuesta Th1 y por tanto facilitando la infección (Choi et al., 2003; Kim et al., 2012). En la misma dirección, estudios realizados en animales de experimentación infectados con *C. sinensis* indican que el incremento de las poblaciones linfocitarias Th2 y Treg juega un papel decisivo en el desarrollo de fibrosis biliar (Zhang et al., 2017).

Aunque se han realizado trabajos para dilucidar el papel de las repuestas Th17 en estas infecciones, se ha visto un incremento de estas poblaciones celulares en ratones infectados con *C. sinensis*. Sin embargo, aún hacen falta más estudios para conocer con exactitud la función específica de estas poblaciones linfocitarias en la defensa contra estos trematodos (Yan et al., 2015).

Mecanismos de evasión

Existen escasos datos experimentales sobre los mecanismos de evasión de trematodos de la familia Opistorchiidae. Sin embargo, estudios del proteoma de *C. sinensis* ponen de manifiesto las diferencias moleculares existentes entre las fases juveniles y los parásitos adultos (Jex et al., 2012). Esta composición y recambio es crucial para desarrollar estrategias de evasión parasitaria.

Por otro lado, se han identificado proteasas y enzimas antioxidantes, altamente expresadas en las fases adultas de estos trematodos. Estas moléculas pueden impedir los ataques inmunológicos del hospedador inactivando los anticuerpos generados por respuestas inmunológicas tipo Th2 (Yoo et al., 2011; Young et al., 2010).

1.5.8 Manifestaciones clínicas

Hasta la fecha, no existen descripciones exhaustivas de la sintomatología causada por la infección de *Amphimerus* spp. A priori, la mayoría de las personas infectadas que hemos observado en las zonas endémicas son asintomáticas (datos observacionales no publicados). Si tenemos en cuenta los datos clínicos de las trematodosis afines como clonorquiosis u opistorquiosis, algunos autores estiman que solo el 5 – 10% de las personas infectadas presentan sintomatología inespecífica como dolor abdominal, flatulencia o fatiga (Pungpak et al., 1983; Upatham et al., 1984). Sin embargo, hay que tener en cuenta que estos síntomas pueden ser ocasionados por otro tipo de parasitosis intestinal presente en zonas endémicas (Vasco et al., 2014).

En un estudio piloto realizado por nuestro grupo de investigación en nueve pacientes con elevada carga parasitaria de *Amphimerus* spp, sólo dos tuvieron un leve aumento de transaminasas hepáticas y un tinte subictérico de mucosa conjuntival. Se realizó ecografía hepática, observando zonas hipodensas en vías biliares intrahepáticas traduciendo una leve dilatación de las mismas (Figura 8). El resto de los pacientes no

presentaron sintomatología o alteración de la función hepática (datos no publicados). Los datos de laboratorio mostraron eosinofilia en tan solo un paciente con un 14,5% de porcentaje de eosinófilos.

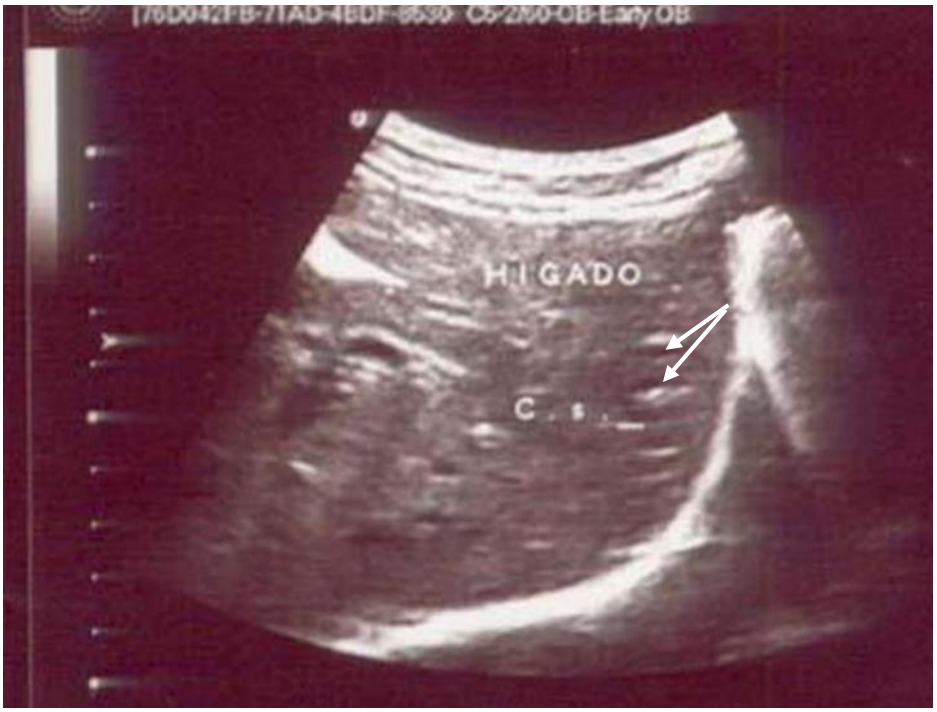


Figura 8. Ecografía hepática de paciente ecuatoriano infectado con *Amphimerus* spp. La ecografía muestra la dilatación leve de vías biliares intrahepáticas en paciente de 47 años de edad procedente de la zona endémica Chachi.

Las manifestaciones clínicas presentes en las trematodosis afines más estudiadas indican que en pacientes con alta carga parasitaria (entre 100-1000 parásitos) los síntomas que pueden presentarse son debilidad generalizada, malestar en epigastrio, parestesias, pérdida de peso, taquicardia, diarrea, vértigo, y disfunción hepática (Belding, 1965; Keiser & Utzinger; 2009). En pacientes con una elevadísima carga parasitaria (>25.000 trematodos) los síntomas incluyen dolor en cuadrante superior derecho, marcada disfunción gastrointestinal, hepatoesplenomegalia, ictericia, ascitis y cirrosis portal (Keiser & Utzinger; 2009; Mas-Coma & Bergues; 1997). En este punto es importante señalar que estudios histopatológicos realizados en gatos y en un cormorán de doble cresta infectados con *Amphimerus* spp. se demostró la presencia de cirrosis hepática y pancreatitis (Rothenbacher & Lindquist; 1963; Pense & Childs; 1972).

No tenemos datos de complicaciones en las infecciones humanas producidas por *Amphimerus* spp. Sin embargo, son bien conocidas las complicaciones de la infección crónica por *C. sinensis,* como colecistitis, colangitis por *Escherichia coli* y más frecuentemente colelitiasis (Qiao et al., 2014). Adicionalmente, pueden presentarse casos de abscesos hepáticos y pancreatitis (Hou, 1955; Kim, 1999). En niños se ha reportado diarrea, inapetencia, malnutrición, anemia y hepatomegalia, lo cual genera retardo del crecimiento (Quian et al., 2016). Pero, sin lugar a dudas, la mayor complicación de las infecciones por *C. sinensis* y *O. viverrini* es el desarrollo de CCA, estando estos parásitos clasificados actualmente como agentes carcinogénicos del grupo 1 por la Agencia Internacional para la Investigación del Cáncer (Bouvard et al., 2009; Sripa, B et al., 2011).

1.5.9 Diagnóstico

1.5.9.1 Métodos parasitológicos

En la actualidad, el diagnóstico de las infecciones en los seres humanos por *Amphimerus* spp. se basa en la observación microscópica directa de huevos del parásito en las heces. La sensibilidad de este método diagnóstico es hasta diez veces menor comparada con la técnica de concentración de formalina-éter (Calvopiña et al., 2011; 2015).

En un estudio preliminar realizado por nuestro grupo se comparó la sensibilidad de cuatro métodos de detección de huevos en heces de *Amphimerus* spp. Los métodos incluyeron la técnica de Kato-Katz, la técnica de sedimentación simple, la concentración con formalina-éter y el examen en fresco mediante microscopía. La sensibilidad de los métodos utilizados fue de 25,7%, 21%, 18% y 1% respectivamente (Calvopiña et al., 2017; *submitted*).

Los huevos de otros parásitos de la familia Opisthorchiidae pueden ser detectados en las heces, lo cual representa la mejor manera de obtener un diagnóstico definitivo, aunque este enfoque se vuelve cada vez menos fiable en casos de cargas parasitarias bajas (Johansen et al., 2010; Kim et al., 2011). Por lo tanto, pueden pasar desapercibidas infecciones debidas a *Amphimerus* spp. o tener dificultades para realizar un diagnóstico en zonas endémicas. El trabajo realizado por Calvopiña y colaboradores (2011) mostró el 3,4% de positividad mediante el método de observación microscópica directa de huevos de *Amphimerus* spp. en heces. Posteriormente y utilizando la técnica de concentración de Ritchie mejoró la sensibilidad hasta alcanzar el 34%. Sin embargo, esta técnica debe realizarse preferiblemente 2 o 3 veces en días diferentes, ya que la eliminación del parásito no se produce de forma continua. Esto justifica el desarrollo de técnicas en etapas tempranas de la infección posibilitando así su diagnóstico precoz.

1.5.9.2 Técnicas inmunológicas

Hasta el momento no se dispone de métodos serológicos que permitan el diagnóstico de la amphimeriosis. Las técnicas inmunológicas, tales como los métodos basados en detección de anticuerpos que utilizan el ensayo inmunoabsorbente ligado a enzimas (ELISA), han mostrado una alta sensibilidad y especificidad para diagnosticar diversas infecciones parasitarias (Elkins et al., 1991; Guevara et al., 1995). De particular importancia es la técnica de ELISA utilizada para detectar parásitos de la familia Opisthorchiidae (Meniavtseva et al., 1996). *Clonorchis sinensis* y *Opistorchis* spp. inducen niveles elevados de IgG en animales experimentales, lo cual es similar a las observaciones en seres humanos (Elkins et al., 1991; Gómez-Morales et al., 2013).

Se han utilizado antígenos de extractos crudos de vermes adultos, aunque con diferentes niveles de sensibilidad y especificidad (Sawangsoda et al., 2012; Poogrushpong et al., 1990). Posteriormente se han empleado antígenos recombinantes derivados de proteínas del tegumento (CsTP20.8) de *C. sinensis*, incrementando la especificidad, pero disminuyendo la sensibilidad de la prueba (Quian et al., 2016). También han sido utilizados antígenos recombinantes derivados de productos de ES de *C. sinensis*, denominado GRCsP (proteína rica en glicina), cuyos resultados han sido altamente específicos y sensibles (Kim et al., 2009).

En un trabajo reciente se han detectado antígenos circulantes de *C. sinensis*, útiles para la evaluación de pacientes que han recibido tratamiento (Nie et al., 2014).

1.5.9.3 Diagnóstico molecular

Como se ha referido en apartados anteriores, el diagnóstico de la infección por *Amphimerus* spp. generalmente se realiza mediante el examen microscópico de las heces y la observación directa de los huevos del parásito (Calvopiña et al., 2011; 2015). Sin embargo, es difícil la diferenciación morfológica con otros huevos de trematodos hepáticos afines como *C. sinensis* u *Opisthorchis* spp. y con trematodos intestinales diminutos (Johansen et al., 2010). Por lo tanto, es necesario el desarrollo de técnicas de diagnóstico directo e indirecto más sensibles y específicas que eviten errores de identificación.

El uso de técnicas moleculares se ha convertido en una herramienta importante para diferenciar especies estrechamente relacionadas. En los últimos años, se han desarrollado varios métodos de diagnóstico molecular basados en la amplificación de ADN mediante la reacción en cadena de la polimerasa (PCR) para la detección de trematodos, incluidas las especies que están estrechamente relacionadas con *Amphimerus* spp., como *C. sinensis* (Parvathi et al., 2007; Kim et al., 2009; Rahman et al., 2011; Cai et al., 2012; Sanpool et al., 2012; Huang et al., 2012) y *O. viverrini*

(Wongratanacheewin et al., 2002; Muller et al., 2007; Lovis et al., 2009; Kaewkong et al., 2013). Aunque estos estudios han demostrado que los métodos basados en la PCR son muy sensibles y específicos, aún no se utilizan de manera rutinaria en países de baja renta ya que se necesita personal capacitado y equipos costosos, lo que los hace inviables para su aplicación en condiciones de campo.

Por otro lado, hay que tener en cuenta que la sensibilidad obtenida mediante la técnica PCR depende en la mayoría de los casos de la calidad del proceso de extracción de los ácidos nucleicos, ya que se conocen diversos inhibidores de la *Taq* polimerasa presentes en las heces. Además, algunos compuestos y derivados de los alimentos (p.e. polifenoles, taninos, terpenos, polisacáridos y resinas) pueden limitar la extracción de ADN (Arimatsu et al., 2012). Así, al realizar la PCR usando ADN extraído de parásitos adultos, donde la contaminación es menor a la de otros compuestos, la reacción se produce con una sensibilidad más elevada, mientras que si la reacción se lleva a cabo con ADN obtenido a partir de heces, la sensibilidad se reduce considerablemente. Por tanto, sería extremadamente difícil aplicar una técnica de PCR en condiciones de campo ya que no se dispone de las condiciones necesarias para la realización de esta técnica en sitios remotos.

Por estas razones, el desarrollo de una técnica de amplificación de ADN en condiciones isotérmicas y de fácil implementación en el campo para el diagnóstico de *Amphimerus* spp. es de gran importancia. En dicho contexto, en el año 2000, fue desarrollada la técnica LAMP (*Loop mediated isothermal amplification*) la cual permite una amplificación rápida y altamente específica de ADN en condiciones isotérmicas (Notomi et al., 2000) (Figura 9). Diferentes estudios han demostrado su elevada sensibilidad y especificidad en diversas enfermedades parasitarias (Aritmasu et al., 2015), incluyendo varias especies de trematodos (Ai et al., 2010; Cai et al., 2010; Chen et al., 2011; Fernández-Soto et al., 2014; Le et al., 2012). La detección visual mediante turbidez o colorimetría y su sencillez en la realización le facilitan su aplicación en condiciones de campo (Njiru, 2010; Mori et al., 2013).

Se ha desarrollado un método LAMP basado en la amplificación de secuencias altamente conservadas del ITS-1 para la detección de *O. felineus, F. gigantica* y *Haplorchis* spp. (Aritmasu et al., 2015). No obstante, sería necesario diferenciar entre trematodos que cohabitan en una misma zona. En este sentido, se ha desarrollado un LAMP frente a *C. sinensis* con alta sensibilidad, amplificando el gen mitocondrial nad1 (Le et al., 2012) o el gen cox1 (Rahman et al. 2017). Además, un estudio usando microsatélites ha permitido diferenciar entre *Opisthorchis viverrini* y *O. felineus* (Aritmasu et al., 2015).

En Ecuador, las infecciones humanas con *Fasciola hepatica* y *Paragonimus mexicanus* son endémicas en la misma zona donde se ha detectado *Amphimerus* spp. Por tanto, sería de gran utilidad el desarrollo de un método LAMP para la detección específica de la amphimeriosis. Además, disponer de un método molecular de fácil aplicación en campo permitiría avanzar en el diagnóstico de esta parasitosis.

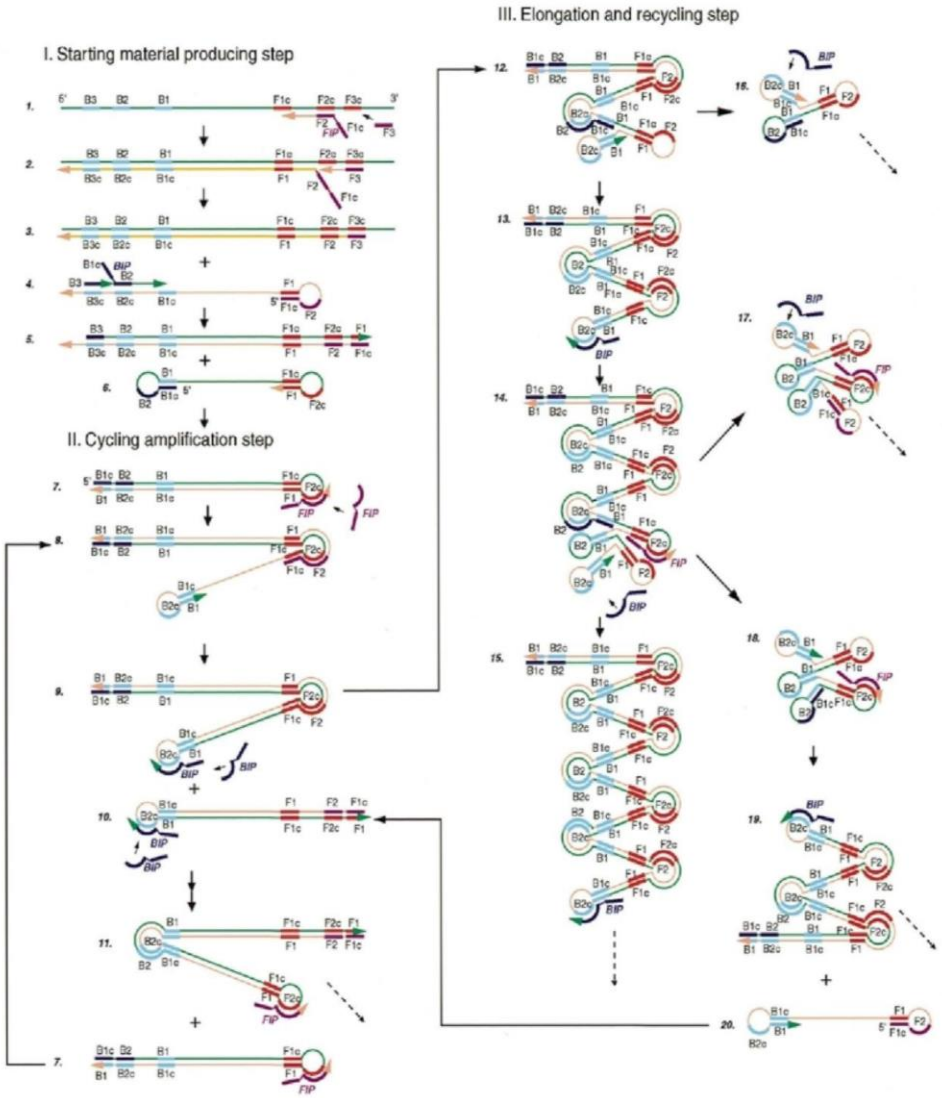


Figura 9. Representación esquemática de la reacción LAMP.

(Imagen tomada de Notomi et al., 2000).

1.5.10 Tratamiento

Es conocido que el fármaco praziquantel (PZQ) destruye los ovarios y testículos de los trematodos inhibiendo la producción de huevos por los parásitos adultos (Lee et al., 1987), aunque su mecanismo preciso de acción es todavía poco comprendido. Tiene un amplio espectro de actividad contra varios trematodos con un excelente perfil de seguridad, lo que le convierte en un fármaco de elección para muchas trematodosis (Keiser & Utzinger, 2004; Utzinger & Keiser, 2004). El PZQ es la droga de primera elección en el manejo y tratamiento de las otras opistorquiosis producidas por *C. sinensis* y *Opisthorchis* spp. Sin embargo, ya se han descrito resistencias en infecciones por *Schistosoma mansoni* y *S. japonicum* (Chai, 2013).

En un estudio piloto realizado por nuestro grupo de investigación se utilizó PZQ a dosis de 25mg/kg de peso, vía oral, tres veces al día durante tres días consecutivos en pacientes con infección por *Amphimuerus* spp., obteniendo altas tasas de curación clínica y parasitológica superiores al 95% (datos no publicados). Son necesarios más estudios para evaluar la dosis, eficacia y posibles efectos adversos. A pesar de que este fármaco consta en el del cuadro nacional de medicamentos básicos del Ecuador (CNMB, 2016), no se encuentra disponible en el mercado ecuatoriano.

1.5.11 Prevención y control.

Las intervenciones en Salud Pública para prevenir las infecciones por trematodos transmitidos por alimentos deben incluir disponibilidad y acceso a la quimioterapia, adecuado saneamiento e higiene, cambio de prácticas de uso y consumo de alimentos y campañas de información, comunicación y educación. La implementación de un control integrado que incluya todas estas medidas comparado con la utilización exclusiva de tratamiento ha sido más eficaz en el control de *O. viverrini* en Tailandia, valorada mediante la tasa de reinfección (Sornmani et al., 1984). En China, la prevalencia de *Paragonimus* spp. fue reducida significativamente sobre todo con la implementación de educación en salud (Blair et al., 2007). Adicionalmente, está bien documentado que la mejora en el saneamiento e higiene influye en la prevalencia de estas enfermedades. Un estudio realizado en Laos mostró una significativa asociación entre opistorquiosis y escaso saneamiento e higiene en poblaciones de bajos recursos económicos (Sayasone et al., 2007).

Los cambios culturales requieren de tiempo y perseverancia con la finalidad de evitar las fuentes de contagio. Lo que se busca es un cambio de actitudes y comportamientos culturales relacionados con la producción, preparación y consumo de alimentos. Otro elemento que se debe tener en cuenta es lograr una seguridad alimentaria que permita a las poblaciones en riesgo poder suplantar las fuentes de

proteína animal en la dieta, siendo una acción necesaria que debe ser considerada por los gobiernos locales y nacionales.

No hay vacunas contra ninguna de las trematodosis, incluida la amphimeriosis. Para su desarrollo es imprescindible llegar a conocer los mecanismos patogénicos involucrados en la infección, así como disponer de la secuenciación del genoma de *Amphimerus* spp. y del conocimiento de su transcriptoma y proteoma.

1.6 Zona geográfica del estudio.

El área de estudio está situada en el noroeste de la República del Ecuador, cantón Eloy Alfaro de la provincia de Esmeraldas (Figura 10). En este Cantón existen alrededor de 150 comunidades. Los pueblos están situados a lo largo de tres ríos, el Río Cayapas, Río Santiago y Río Onzole, ríos que desembocan en el estuario de la ciudad de Borbón, y que reciben un sinnúmero de afluentes. Borbón es la ciudad más grande del área con cerca de 5.000 habitantes. En total, aproximadamente 25.000 personas residen en la región.

El estudio se realizó en tres comunidades indígenas Chachis a lo largo del río Cayapas (Figura 11). Esta área forma parte del bosque lluvioso tropical llamado "Chocó biogeográfico del Pacífico" que cubre parte de las costas de Ecuador, Colombia y Panamá. Esta área ha sido catalogada como un "*biological hotspot*" (Myers et al., 2000), es decir, una zona considerada de alta biodiversidad pero en peligro de extinción por la rápida pérdida de sus recursos naturales. El clima es cálido y húmedo, con una temperatura promedio de entre 24°C y 28°C y una humedad relativa promedio del 85% (Sierra, 1999).

De acuerdo al censo nacional ecuatoriano del 2010, casi un 55% de la población se autodefine como afroecuatoriano y un 13% como Chachi, el grupo indígena que predomina en la región (Instituto Nacional de Estadísticas y Censo, 2001). Los demás se autodefinen como mestizos, mulatos, blancos, cholos, categorías que refieren a tendencias migratorias recientes provenientes de otras provincias del país (Minda, 2002). En general, los pueblos lejanos a Borbón tienen mayores porcentajes de afroecuatorianos, excepto en los altos de los Ríos Cayapas y Onzole donde predominan los Chachis. El promedio de años de educación varía entre 3,1 y 6 (Trostle et al., 2008).

Figura 10. Localización geográfica del área de estudio en la provincia de Esmeraldas, Ecuador, a 320 km desde la capital Quito y a 127 a 132 km del borde del Océano Pacifico.

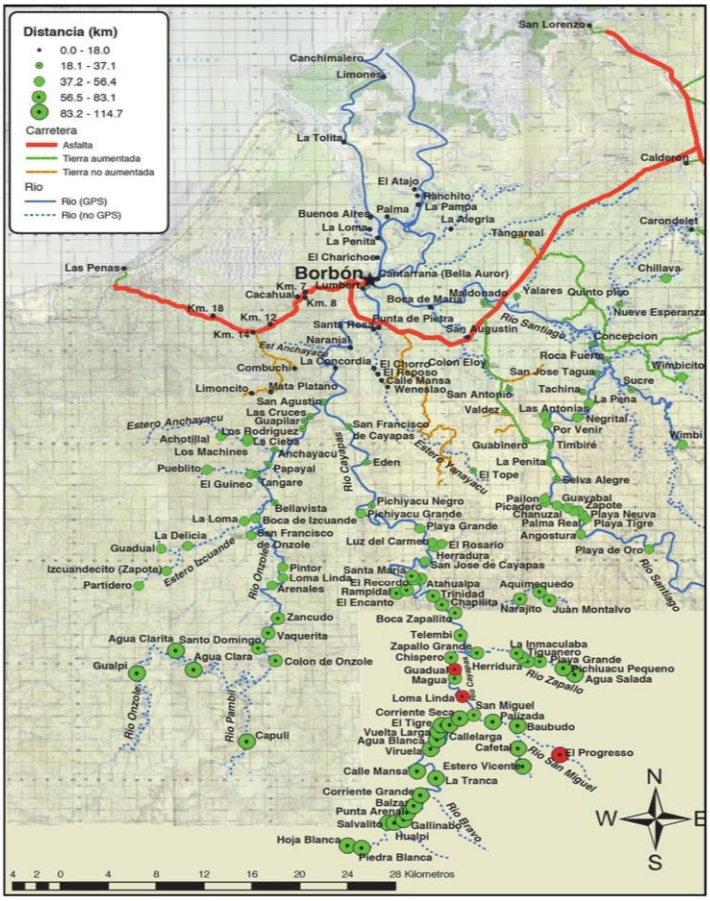


Figura 11. Mapa del área donde se muestran en círculos rojos las 3 comunidades estudiadas a lo largo del Río Cayapas y su afluente Río San Miguel.

Los indígenas Chachis viven en comunidades remotas donde la única forma de llegar es por canoa a lo largo del río (Figura 12). Mantienen una infraestructura poco desarrollada con aguas residuales no tratadas que son eliminadas detrás de las casas, rudimentarios sistemas de eliminación de residuos sólidos. Una de las principales fuentes alimenticias es el pescado y crustáceos recolectados en los ríos principales y sus afluentes.

Figura 12. Vista panorámica del acceso a las comunidades del Río Cayapas.

La fuente de su agua es principalmente de ríos y arroyos y la consumen sin tratamiento, aunque el agua de lluvia se utiliza de manera intermitente; algunas comunidades tienen pozos o reciben agua corriente de fuentes superficiales. Las instalaciones de saneamiento son de calidad variable, aunque generalmente se clasifican como no mejorados mediante criterios de la Organización Mundial de la Salud; los inodoros son poco frecuentes.

Las personas son cazadoras y comen pescado poco cocinado y capturado en los ríos vecinos casi todos los días y la comida se acompaña con arroz cocido y plátano (Figura 13). Mayor información de la zona de estudio y sus habitantes se encuentra muy bien detallada en la literatura (Whitten, 1965; 1974; Sierra, 1998; 1999).

Figura 13. Forma de preparación y consumo de alimentos en la población Chachi.

1.7 Bibliografía

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2. HIPÓTESIS Y OBJETIVOS

2.1 Hipótesis

De la revisión bibliográfica podemos extraer las siguientes conclusiones: *(i)* aunque la amphimeriosis ha sido descrita en casos aislados, se desconoce actualmente su situación epidemiológica y, *(ii)* los métodos de diagnóstico parasitológicos clásicos son insuficientes para conocer el alcance real de esta infección.

Por lo tanto, la hipótesis de esta tesis doctoral es probar que la amphimeriosis es una zoonosis prevalente en comunidades con hábitos alimentarios de consumo de peces de agua dulce crudos o insuficientemente cocinados. Para ello es necesario disponer de nuevas herramientas más útiles para su diagnóstico.

2.2 Objetivo general

Estudio epidemiológico y desarrollo de técnicas de diagnóstico inmunológico y molecular para la evaluación de la amphimeriosis en Ecuador.

2.2.1 Objetivos específicos

1. Determinar la prevalencia de la infección por *Amphimerus* spp., en población indígena residente en zonas del noroeste de Ecuador.
2. Establecer si la amphimeriosis es una zoonosis, mediante el análisis de muestras de heces de animales domésticos en la misma zona de estudio.
3. Desarrollar, estandarizar y aplicar una técnica diagnóstica basada en el análisis inmunoenzimático tipo ELISA que permita la detección de IgG específicas anti-*Amphimerus* spp. en sueros humanos.
4. Diseñar, desarrollar y evaluar un método molecular tipo LAMP para la detección de ADN de *Amphimerus* spp. en muestras de heces humanas.
5. Definir la viabilidad del método LAMP para la detección de *Amphimerus* spp. en muestras de heces utilizando papel de filtro como soporte.

3. ARTÍCULOS DE INVESTIGACIÓN

3.1 ARTÍCULO 1: High prevalence of human liver infection by *Amphimerus* spp. Flukes, Ecuador.

Manuel Calvopiña, William Cevallos, Joseph Eisenberg.

Emerging Infectious Diseases Vol. 17. No. 12. 2011.

RESUMEN

Es conocido que el trematodo *Amphimerus* spp. infecta a mamíferos, pero las infecciones en humanos aún no han sido confirmadas. La microscopía de muestras fecales de 297 personas de Ecuador, reveló la presencia de huevos de la familia Opisthorchiidae en 71 (24%) personas. La microscopía óptica de parásitos adultos y la microscopía electrónica de barrido de huevos fueron compatibles con las descripciones de *Amphimerus* spp. Este patógeno solo se observó en las comunidades que consumieron pescado mal cocinado.

High Prevalence of Human Liver Infection by *Amphimerus* spp. Flukes, Ecuador

Manuel Calvopiña, William Cevallos, Hideo Kumazawa, and Joseph Eisenberg

Amphimerus spp. flukes are known to infect mammals, but human infections have not been confirmed. Microscopy of fecal samples from 297 persons from Ecuador revealed *Opisthorchiidae* eggs in 71 (24%) persons. Light microscopy of adult worms and scanning electron microscopy of eggs were compatible with descriptions of *Amphimerus* spp. This pathogen was only observed in communities that consumed undercooked fish.

The genus *Amphimerus* Barker 1911 infects mammals from the Americas, including Canada, the United States, Costa Rica, Panama, Colombia, Brazil, and Peru. Eleven species are reported (*1*–*7*). In Ecuador, a trematode resembling *Amphimerus* spp. but identified as *Opisthorchis guayaquilensis* has been reported (*8*,*9*).

Amphimerus spp. are parasitic liver flukes in the bile ducts of mammals, birds, and reptiles (*1*). Although these digenetic trematodes of the *Opisthorchiidae* family are closely related to the genera *Clonorchis* and *Opisthorchis*, there are morphologic differences. The vitellaria in adult *Amphimerus* spp. trematodes are distributed in 4 groups, 2 anterior and 2 posterior; the latter groups extend beyond the posterior testis; the ventral sucker is larger than the oral, and the testes are rounded or slightly lobulated. In contrast, the vitellaria in *Clonorchis* and *Opisthorchis* spp. trematodes exist only in front of the testes. Additionally, *Clonorchis* spp. trematodes have 2 large highly branched testes; testes in *Opisthorchis* spp. worms are always lobulated (*1*,*2*). The eggs of the flukes from these genera can be differentiated only by using scanning electron microscopy (SEM). Definitive diagnosis using light microscopy of flukes of the *Opisthorchiidae* family, therefore, is not possible unless the adult worm is collected and identified. Through isolation of adult worms and SEM of eggs, we found a high prevalence of human infection with a trematode of the genus *Amphimerus* in Ecuador.

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The Study

In June 2009, during a routine fecal examination for the parent study, 4 samples tested positive for eggs of the *Opisthorchiidae* family in 3 indigenous Chachi communities along the Cayapas River in the northern coastal rainforest of Ecuador. In January 2010, a follow-up survey was conducted in the same 3 communities (total population 589); all villagers, whether symptomatic or not, were asked to provide a fecal sample. Specifically, a community meeting was held in each village, study objectives were explained, and villagers were asked for their voluntary participation. Flasks were distributed to all villagers and collected the next day in the school and by going house to house. The Chachis, the predominant group in these 3 communities, represent 13% of the 24,000 inhabitants in the region. Afro-Ecuadorians and mestizos also reside in this region (*10*,*11*).

A total of 297 (50.4%) community members 3–77 years of age provided samples. To each person providing a sample, a questionnaire was administered regarding types of food eaten and cooking practices. Samples were preserved in 10% formalin, transported to a laboratory in Quito, and stored at 4°C until examination by light microscopy. Eggs were concentrated by using the formalin-ether technique. In addition, 120 fecal samples from Afro-Ecuadorian and mestizo persons were examined. The villagers were informed of the study in their local Chapalache language by community health community workers. The ethical committee of the Central University approved this study.

Duodendoscopy was performed in 4 patients by a gastroenterology specialist to examine the biliary liquid; the microscopy of this liquid showed eggs identical to those found in their feces. These patients received praziquantel (75 mg/kg in 3 doses/3 d), and were purged with 10 mg of bisacodilo. Fecal samples were collected and examined for worms as previously described (*12*). Recovered worms were fixed with 10% formalin, stained with Diff-Quik fixative (Sysmex, Kobe, Japan), and identified by comparing their morphologic features to known adult *Clonorchis* and *Opisthorchis* spp. worms. Community health workers collected and examined the livers of 3 cats and 3 dogs from 1 of the 3 communities. All 6 livers had eggs and high numbers of adult parasites in the bile ducts. Adult parasites were stained, and microscopic studies showed them to be identical to those in the human specimens.

A total of 71 (24%) of the 297 fecal samples from the indigenous Chachi were positive for *Opisthorchiidae* eggs (Table). In contrast all 120 samples from Afro-Ecuadorian and mestizo persons were negative. Eggs

were yellow-brown and measured 28–33 μm ×12–15 μm (n = 20). The operculum and the shoulders, however, were not prominent as they are in *Clonorchis* and *Opisthorchis* eggs. Occasionally, a small knob, but most frequently a curved spine, was seen on the abopercular end. Although, by light microscopy, the shape and size of the eggs resembled that of the other liver flukes, the patterns of the eggshell surface were distinct when viewed with SEM (Figure 1). This observation is corroborated with published photographs (*3*).

After participants were treated with praziquantel, a total of 8 worms were recovered from 4 human participants and dozens from cat and dog livers; all were placed in saline. The worms were delicate, leaf-shaped, elongated, and red-pink and measured 8–13.6 mm long (average 10.2 mm) × 0.5–1.1 mm wide (n = 15). After a few minutes, the worms coiled in an S shape and became transparent or whitish. Once stained, the following features were observed: 1) the vitellaria divided into an anterior and posterior group with the posterior group extending the level of the posterior testis; 2) a ventral sucker larger than oral sucker; and 3) 2 rounded testes (Figure 2). On the basis of these morphologic characteristics of the adults and the SEM findings of the eggs, the parasite was identified as *Amphimerus* spp.

Conclusions

Our study demonstrates that the liver fluke of the genus *Amphimerus* can infect humans. We found a high prevalence (15.5%–34.1%) of infection with *Amphimerus* spp. trematodes in the surveyed communities (Table). Samples from the Afro-Ecuadorian and mestizo population were all negative for *Opisthorchiidae* eggs. *Amphimerus* spp. trematodes are believed to be transmitted, as are the other members of the *Opisthorchiidae* family, by ingestion of raw or undercooked fish (*2*). In our survey, most Chachis reported eating smoked fish caught in the rivers. Food sharing is more common among Chachi than Afro-Ecuadorians and mestizo families (*13*). Notably, the most remote village (120 km inland from the coast) had the highest prevalence. Our results suggest that *Amphimerus* spp. flukes are zoonotic pathogens of domestic animals living with humans.

Table. Prevalence of *Amphimerus* eggs in feces in 3 villages, Ecuador

Village	Total population	No. samples examined	No. (%) positive	Distance to the coast, km
1	116	82	28 (34.1)	120
2	248	86	23 (26.7)	91
3	253	129	20 (15.5)	85
Total	617	297	71 (23.9)	

Amphimeriasis should be considered an endemic liver fluke infection among residents of this Chachi population in Ecuador. Further studies are needed to determine the complete epidemiology and geographic distribution of infection in this region, as well as in other provinces of Ecuador where freshwater fish is eaten undercooked or where the same tropical ecology is found. For example, the Amazonian region has indigenous groups where other foodborne trematodiasis-like paragonimiasis are endemic (*14*). *Amphimerus* spp. flukes infecting domestic and wild animals have been reported from Ecuador's neighboring countries as well as from Central and North America. The existence of undiscovered foci of human infections is possible.

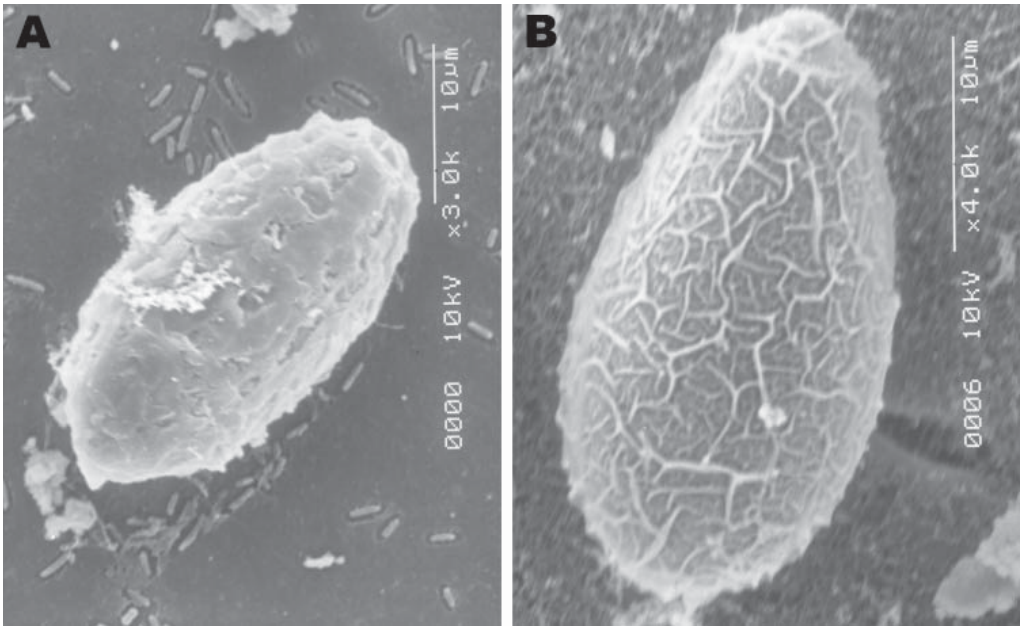

Figure 1. Scanning electron microscopy images of A) an egg of the Ecuadorian *Amphimerus* spp. trematode (original magnification ×3) obtained from a human and B) an egg of the Asian *Clonorchis sinensis* trematode (original magnification ×4). Although the size is similar, the pattern of the surface is different, thus differentiating the 2 genera.

Figure 2 . *Amphimerus* spp. adult trematode (10.1 mm) recovered from a human, Ecuador.

In 1971, Yamaguti (*1*) suggested that a parasite previously reported in Ecuador (*8*) as *O. guayaquilensis* might in fact be *Amphimerus* spp. Subsequently, publications referred to this parasite as *A. guayaquilensis* (*5*,*7*); however, the accuracy of this reclassification is unclear. Molecular analysis could help clarify the ambiguities in genus/species identification of *O. guayaquilensis* parasites and the conspecific species of *Amphimerus* (*15*).

We have much to learn about the pathology and epidemiology of *Amphimerus* spp. flukes. For example, nothing is known about the clinical and pathologic significance of infections with this parasite. Praziquantel eliminated the parasites in these patients, but whether the dose and treatment time were adequate are unknown. Additionally, little is known about epidemiologic factors responsible for the differences in the number of infections among the different population groups. Future studies can help determine the direct and indirect public health implications of this new foodborne zoonosis.

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3.2 ARTÍCULO 2: High prevalence of the liver fluke *Amphimerus* spp. in domestic cats and dogs in an area for human amphimeriasis in Ecuador.

Manuel Calvopiña, William Cevallos, Richard Atherton, Matthew Saunders, Alexander Small, Hideo Kumazawa, Hiromu Sugiyama.

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RESUMEN

Recientemente, hemos demostrado que *Amphimerus* spp. es un parásito trematodo de vías biliares, el cual presenta una alta prevalencia de infección entre el grupo indígena Chachi, residente en la selva tropical, ubicada en el noroeste de la provincia de Esmeraldas, Ecuador, Sudamérica. Al momento, desconocemos los animales que pueden actuar como reservorios y/u hospedadores definitivos para *Amphimerus* spp. En esta zona endémica, se realizó un estudio para determinar la prevalencia de infección en gatos domésticos y perros. Esta información es de vital importancia para entender la epidemiología, el ciclo de vida y el control de este parásito.

En julio de 2012, se realizaron encuestas en tres comunidades Chachis localizadas en el Río Cayapas, provincia de Esmeraldas, Ecuador. Un total de 89 de los 109 hogares registrados participaron en el estudio. De los 27 gatos y 43 perros encontrados que vivían en las comunidades, se recogieron muestras de heces de 14 gatos y 31 perros. Se examinó microscópicamente la presencia de huevos de *Amphimerus* spp. La prevalencia de infección fue de 71,4% en gatos y 38,7% en perros, con índices similares de infección en las tres comunidades. Significativamente más gatos que perros fueron los infectados por el parásito ($p = 0.042$).

Los datos muestran una alta tasa de infección por *Amphimerus* spp. en gatos domésticos y perros que residen en las comunidades Chachis. Se puede concluir que estos animales actúan como hospedadores definitivos y reservorios del parásito y por tanto que la amphimeriosis es una enfermedad zoonótica. Estos hallazgos proporcionan importantes datos epidemiológicos que ayudarán en el desarrollo e implementación de estrategias de control contra la transmisión de *Amphimerus*.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

High Prevalence of the Liver Fluke *Amphimerus* sp. in Domestic Cats and Dogs in an Area for Human Amphimeriasis in Ecuador

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Data Availability Statement: All sequences of the ribosomal DNA ITS2 region of the flukes are available in GenBank (Accession numbers, deposited in the GenBank/EMBL/DDBJ nucleotide database, are AB678442, AB926429 and AB926430 for *Amphimerus sp* from humans, cats and dogs, respectively.)

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Abstract

Background

Amphimerus sp. is a liver fluke which recently has been shown to have a high prevalence of infection among an indigenous group, Chachi, who reside in a tropical rainforest in the northwestern region of Ecuador. Since it is unknown which animals can act as a reservoir and/or definitive hosts for *Amphimerus* sp. in this endemic area, a study was done to determine the prevalence of infection in domestic cats and dogs. This information is important to understand the epidemiology, life cycle and control of this parasite.

Methodology/Findings

In July 2012, three Chachi communities located on Rio Cayapas, province of Esmeraldas, were surveyed. A total of 89 of the 109 registered households participated in the study. Of the 27 cats and 43 dogs found residing in the communities, stool samples were collected from 14 cats and 31 dogs (total of 45 animals) and examined microscopically for the presence of *Amphimerus* eggs. The prevalence of infection was 71.4% in cats and 38.7% in dogs, with similar rates of infection in all three communities. Significantly more cats were infected than dogs ($p = 0.042$).

Conclusions/Significance

The data show a high rate of *Amphimerus* sp. infection in domestic cats and dogs residing in Chachi communities. It can be concluded that these animals act as definitive and reservoir hosts for this liver fluke and that amphimeriasis is a zoonotic disease. These findings provide important epidemiological data which will aid in the development and implementation of control strategies against the transmission of *Amphimerus*.

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Author Summary

Amphimerus sp. is a fluke that infects the bile ducts of its definitive hosts. Recently, it has been shown that an indigenous Amerindian group, the Chachi, living in a rural and remote tropical area of Ecuador, are infected with this parasite. The epidemiology and life cycle of this parasite remains elusive, and research is needed to understand the mode of transmission and zoonotic potential of the parasite. It was hypothesized that the domestic animals of the Chachi households may act as definitive and reservoir hosts for *Amphimerus* infection. Hence, the presence and prevalence of infection in these animals residing in communities endemic for human amphimeriasis was investigated. Some 45 animal stool samples were examined microscopically for the presence of *Amphimerus* eggs. The results showed an infection rate of 71.4% in cats and 38.7% in dogs. The data provided evidence that these domestic animals act as both definitive and reservoir hosts for the parasite and that amphimeriasis is a zoonotic disease. The implementation of a mass treatment/control program must target both humans and animals in order to minimize the transmission of this liver fluke.

Introduction

Amphimerus Barker, 1911 is a genus of parasitic liver fluke which are flat helminths (Platyhelminthes) of the Trematoda class and belong to the Opisthorchiidae family. The adult flukes reside within the bile ducts of a definitive host [1]. Infection by liver flukes of this family, which include *Clonorchis sinensis* and *Opisthorchis* spp. can occur through the consumption of raw or undercooked, metacercariae infected freshwater fish [1–5]. Liver fluke infection is one of the more important food-borne diseases worldwide and is considered by the World Health Organization as a neglected tropical disease [6]. Affected individuals with liver flukes of the Opisthorchiidae family can suffer from suppurative cholangitis, cholelithiasis and cholangiocarcinoma [3,5,6].

In the Americas, ten species of *Amphimerus* which infect mammals have been described: *A. pseudofelineus*, *A. pseudofelineus minutus*, *A. caudalitestis*, *A. price*, *A. lancea*, *A. parciovatus*, *A. bragai*, *A. minimus*, *A. neotropicalis* and *A. ruparupa* [7,8]. In Ecuador, flukes found in the bile ducts of dogs were previously described as *Opisthorchis guayaquilensis* [9] but later, this species, without any further comparative studies was named as being *Amphimerus guayaquilensis*. However, Artigas and Perez (1964) considered to *A. guayaquilensis* to be synonym of *A. pseudofelineus*. A few years later, *A. guayaquilensis* was considered to be distinct from *A. pseudofelineus* and was instead regarded as a synonym of *A. parciovatus* [7]. Furthermore, Thatcher (1970) did not agree with this synonymy and contemplated *A. guayaquilensis* distinct from *A. pseudofelineus* because of the extent of the vitellaria. The validity of some species in this genus is controversial, since speciation is based only on morphological and morphometric features present in the adult flukes and the assignment of species names must be regarded as speculative.

Amphimerus spp. have been demonstrated to infect birds, reptiles and certain mammals including cats, dogs, ducks, the double-crested cormorant, Amazonian dolphins, opossums (*Didelphis marsupialis*, *Philander opossum*) and the rodent *Nectomys squamipes* [1,9–16]. For other members of the Opisthorchiidae family known to infect humans e.g. *C. sinensis* and *Opisthorchis* spp., cats and dogs are the most important animal reservoirs for human infection [2,3,17,18]. However, it is currently unknown whether domestic animals may act as a definitive and/or reservoir host for human transmission in the recently reported focus of infection in

Ecuador [10]. Given the similarities between *C. sinensis*, *Opisthorchis* spp. and *Amphimerus* spp., it was hypothesized that these mammals may also act as reservoirs for *Amphimerus* sp. infection in Ecuador.

The indigenous group, Chachi, who live along the Rio Cayapas and its tributaries in the north-western coastal rainforest of Ecuador, have been shown to have a high prevalence of infection (15.5% to 34.1%) with *Amphimerus* sp. [10]. Afro-Ecuadorian and mestizo populations living in separate communities but along the same rivers were not found to be infected [10]. The solo infection of the Chachi population was postulated to be related to their cultural tradition of eating smoked fish and food sharing [10,11].

In a previous pilot study, conducted by the authors, in the same endemic communities for human infection, both cats and dogs were found to be positive for *Amphimerus* eggs. There was no evidence of infection in any other animals investigated (e.g. pigs and chickens). The objective of this study was, therefore, to investigate the prevalence of infection of *Amphimerus* sp. in domestic cats and dogs and to determine their role in the transmission in the Ecuadorian villages endemic for human infection.

Materials and Methods

Study area

The study was conducted in three indigenous Chachi communities along the Rio Cayapas in the Province of Esmeraldas, located in the northwest coastal rainforest of Ecuador (Fig. 1). The indigenous Chachi, living alongside Afro-ecuadorian and mestizo populations, is the predominant ethnic group in this area, and represent 13% of the inhabitants in this region of Ecuador [19,20]. These communities are the same as those studied previously, showing the resident Chachi having a high prevalence of infection with *Amphimerus* sp. [10]. They live in remote villages where the only way to reach the communities are by boat along the river; the source of their water is mainly from rivers and streams and is consumed untreated. Sanitation facilities are lacking, and flush toilets are uncommon. The people are hunters and eat fish caught in the neighboring rivers almost every day and the meal is accompanied with cooked rice and plantain.

The province of Esmeraldas, forms part of the tropical rainforest known as "Choco Biogeográfico del Pacífico" which covers a section of the coast of Ecuador, Colombia and Panamá. This area has been labelled as a biological hotspot; an area with an extraordinary concentration of animal species [21]. The climate of this region is warm and humid, with an average temperature of between 24°C and 28°C and an average relative humidity of 85% [22].

Sampling

This study was based on a previous census conducted in January 2012 where each household was given an identification number and a total of 109 households were recorded in the three communities. In July 2012, all house owners were asked to participate in the study by providing a stool sample from any cats and/or dogs residing in the respective household, simultaneously a census of dogs and cats residing in the participating communities was conducted. A team of community health workers informed the villagers of the study in their local Chapalache language and residents were free to refuse entry to their household or access to their domestic animals at any point during data collection.

Sample processing

Stool samples were collected by their owners from each animal directly after the deposit was made. Plastic flasks containing stool samples were labelled with type of animal, house code and

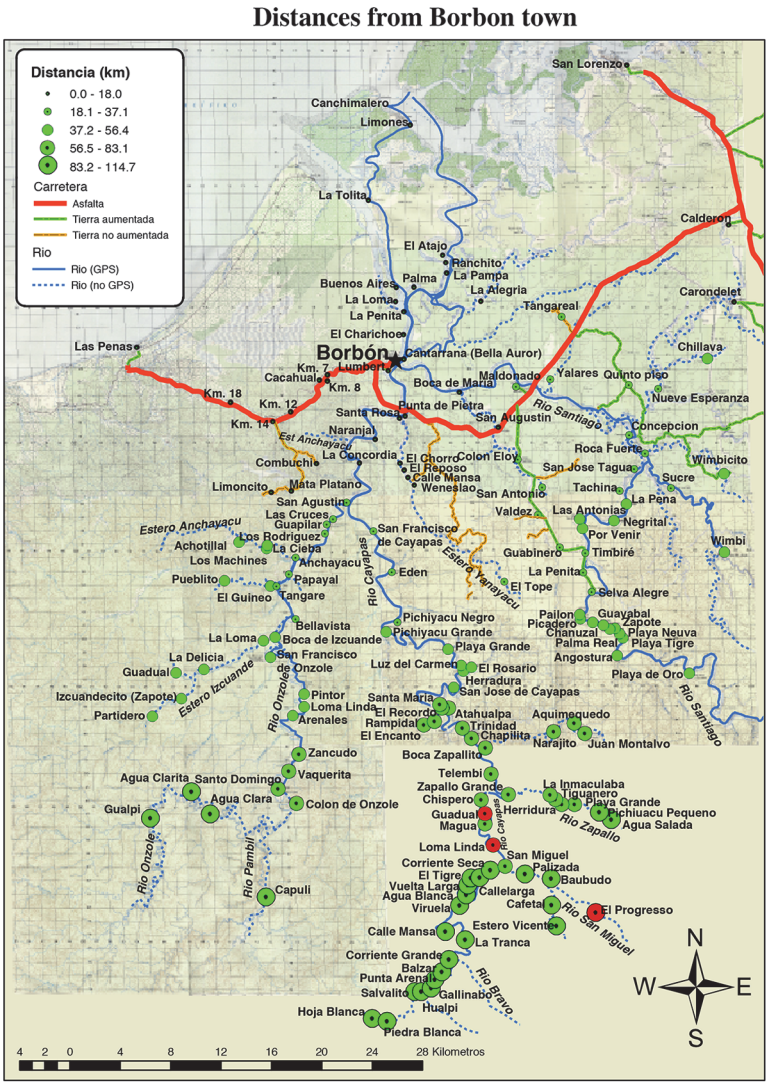

Fig 1. Map of the study area. It showed the geographical location of the study area in the Canton Eloy Alfaro, province of Esmeraldas, 320 km from the capital Quito. In red circles are the 3 communities studied along the Rio Cayapas and its tributary Rio San Miguel.

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date. The samples were preserved in 10% formalin and transported to the parasitology laboratory at Centro de Biomedicina in Quito where they were stored at 4°C until they were examined. Samples were concentrated using the formalin-ether technique as previously described [10] and were examined under light microscopy by at least two laboratory technicians for the presence of *Amphimerus* eggs. Samples were then verified by an expert in animal stool examination who was not involved in the data collection. The primary outcome variable was *Amphimerus* infection, defined as positive if eggs of the parasite were visualized by light microscopy. The yellow-brown eggs measured 20 to 32 *u*m in length and 14 to 16 *u*m in width; they are pyriform with a visible operculum at the narrower anterior end. In the centre of the posterior end there is a small bud (Fig. 2).

The adult parasites were obtained from the livers of two cats and one dog. Animals were presented by their owners as sick and showed severe emaciation. They were euthanized with ether and necropsied. The livers were collected in saline solution, squeezed and sliced in small

Fig 2. An egg of *Amphimerus* sp. observed in stools from a cat. It is seen to be morphologically similar, using light microscopy, to eggs of *Clonorchis sinensis* and *Opisthorchis* spp. (dimensions 31 µm × 15 µm).

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pieces and maintained for 30 minutes. Flukes were then removed from the saline and were fixed in both 70% ethanol and 10% formalin.

Molecular characterization

For molecular analysis, genomic DNA samples were extracted from each of the *Amphimerus* adult specimens from the cats and dogs using a DNeasy Blood & Tissue Kit (QIAGEN K. K., Tokyo, Japan). The ITS2 region of the ribosomal DNA was then amplified by PCR using Ex Taq DNA polymerase (Takara Bio, Shiga, Japan). The primers used were 3S (forward, 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′) [23] and A28 (reverse, 5′-GGGATCCTGGT-TAGTTTCTTTTCCTCCGC-3′) [24]. DNA sequencing of amplicons was performed with a 3100-Advant Genetic Analyzer (Life Technologies, Foster City, CA, USA).

Statistical analysis

Data was analysed using SPSS version 19 (Statistical Product and Service Solutions, Chicago, IL, USA). The data was stratified by village and animal species, and prevalence of *Amphimerus* infection in the animals calculated for each village. A chi squared test was used to detect any significant differences in the prevalence of infection between animal species and between villages.

Ethics

Ethical approval of the study was given by ethic committee of the Universidad Central del Ecuador (licence number LEC IORG 0001932, FWA 2482, IRB 2483.COBI-AMPHI-0064–11). All villagers were asked for their verbal consent to access their domestic animals and collect stool samples. Infected animals with any parasite were treated with specific drugs free of charge in the following months. The study was conducted according to the above institution's guidelines for animal welfare.

Results

Of the 109 houses recorded in the communities, 89 (81.6%) agreed to take part in the survey (Table 1). From these 89 houses, a total of 27 cats and 43 dogs were counted at the time of data collection. Stool samples from 45/70 animals (64.2%), 14 from cats and 31 from dogs were collected and examined microscopically for the presence of *Amphimerus* eggs (Table 1). Samples of the remaining 25 animals were not able to be collected because they either did not defecate on the collection day or were not present in the home at the time. In cats the prevalence of *Amphimerus* infection was 71.4% (95% confidence interval [CI] = 47.7–95.1), and in dogs, 38.7% (95% CI = 21.6–55.8) (Table 1). The overall prevalence of *Amphimerus* sp. in the two animal species investigated was 48.9% (95% CI = 34.3–63.5). Cats were approximately four times more likely to be infected with *Amphimerus* sp. than dogs, OR = 3.95 (95% CI 1.01–15.6, p = 0.042). There was no statistically significant difference between the communities with regard to prevalence of *Amphimerus* infection in the animals studied.

The eggs found in the stools of cats and dogs demonstrated the morphological characteristics consistent with other members of the Opisthorchiidae family (Fig. 2). In order to confirm that the eggs were from *Amphimerus* sp., two positive cats and one positive dog were euthanized and adult flukes were obtained from the bile ducts and subjected to morphological and molecular characterization. The recovered flukes just after extraction from the bile ducts were flat, leaf-like, reddish, flexible and elongated with active movements in saline, with a thinner anterior than posterior extremity, measuring from 15 to 28 mm in length by 2 to 4 mm in width. When fixed in formalin 10%, the flukes became whitish and shorter measuring around 10 to 18 mm long. Flukes were stained with borax carmine and photographed (Fig. 3). Adults of *Amphimerus* sp. can be differentiated from *Clonorchis sinensis* and *Opisthorchis* spp. by certain morphological features e.g.: 1) The presence of two rounded testes, which lie one behind the other in the posterior portion, 2) The vitellaria occupy both lateral sides of the fluke,

Table 1. Demographics of three villages in northwest Ecuador and the prevalence of *Amphimerus* sp. infection in domestic cats and dogs of these villages.

Village	No. of houses		No. of dogs			No. of cats		
	Total	Surveyed (%)	Total	Examined (%)	Positive (%)	Total	Examined (%)	Positive (%)
1	22	17 (77.2)	10	8 (80)	3 (37.5)	8	4 (50)	3 (75)
2	44	32 (72.7)	19	13 (69)	6 (46.1)	10	5 (50)	3 (60)
3	43	40 (93)	14	10 (72)	3 (30)	9	5 (56)	4 (80)
Total	109	89 (81.6)	43	31 (72)	12 (38.7)	27	14 (52)	10 (71.4)

List of accession numbers/ID numbers for the sequences of the ribosomal DNA ITS2 region of the flukes obtained from humans, cats and dog
Accession numbers, deposited in the GenBank/EMBL/DDBJ nucleotide database are: AB678442 for *Amphimerus* sp. from humans
AB926429 for *Amphimerus* sp. from cats
AB926430 for *Amphimerus* sp. from a dog.

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Fig 3. Adult fluke obtained from the biliary ducts of a cat. The major difference of *Amphimerus* flukes from the others Opisthorchiidae is that the vitelline glands situated along both lateral sides of the body are divided into anterior and posterior clusters at the level of the ovary, distributed in four groups, 2 anterior and 2 posterior extending nearly to the excretory pore.

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outside of the intestinal branches and are conspicuously distributed in four groups, 2 anterior and 2 posterior extending backwards nearly to the excretory pore, and 3) the ventral sucker is larger than the oral [1,10]. Furthermore, sequences of the ribosomal DNA ITS2 region of the flukes obtained from the cats and dog were identical to that obtained from humans in the previous study [10]. Accession numbers, deposited in the GenBank/EMBL/DDBJ nucleotide database, are AB678442, AB926429 and AB926430 for *Amphimerus* sp. from humans, cats and dogs, respectively.

Discussion

The current study is the first to identify *Amphimerus* sp. infection in the domestic animals from Chachi communities in which high rates of infection have been demonstrated [10]. The overall prevalence of *Amphimerus* sp. infection in cats and dogs was relatively high and it can be concluded that these species act as definitive hosts for *Amphimerus* sp. in the study region. As the eggs and adult flukes were demonstrated to be morphologically similar and molecularly identical to those obtained from humans it can be further assumed that they act as reservoir hosts for human infection. This confirms that amphimeriasis is a zoonosis from domestic animals living together with humans. Importantly, the prevalence of infection in the cats and dogs recorded in this study was significantly higher than in the humans of the same communities (48.9% in animals compared to 24% in humans) [10]. These findings suggest that these animals play a role in the transmission of *Amphimerus* to humans. It is of interest to note that the animals studied have contact with villages inhabited by Afro-Ecuadorians and mestizo groups and defecate in and contaminate the community streams, thus represent a risk to these populations which were not found to be infected in the previous human study [10].

The prevalence reported here for *Amphimerus* sp. infection in cats and dogs is similar to that of other members of the Opisthorchiidae family. In China and Korea, the average prevalence of *C. sinensis* infection in cats and dogs ranged from 64.1% to 73.2% and from 56.4% to 69.4%, respectively [2]. Furthermore, in a study of *O. viverrini* infection in four districts of Thailand, cats had a much higher prevalence (35.5%) than dogs (0.37%) [18].

Amphimerus spp. flukes are thought to be transmitted by ingestion of raw or undercooked freshwater fish [1–5]. This was previously shown in 2011, when most of the Chachi included in the study, admitted eating smoked fish caught in nearby rivers [10]. There are two possibilities

how these domestic animals can become infected. Firstly, cats and dogs could ingest the leftovers of smoked fish containing the metacercariae of *Amphimerus* sp. Secondly, the animals themselves may swim in the river and feed, thus acquiring infection directly from live fish. The prevalence of *Amphimerus* sp. in cats was significantly higher than in dogs. This is probably because of a cat's preference for fish and increased capability to catch them in streams. Both cats and dogs have been observed fishing and eating leftovers of human food. Overall 230 freshwater fish from the Cayapas River are currently being examined for metacercariae. A number of trematode metacercariae have been identified but none were *Amphimerus*. It is presumed the intermediate host is only found in the rainy season or the natural infection is very low.

Though the study was successful in identifying a zoonotic link between domestic animals and humans, there are some important limitations that indicate the need for further research in order to validate the findings. Firstly, a total sample size of 45 is small and although this did account for over 64% of all cats and dogs living in the three communities, a larger sample is required to obtain a more accurate estimate of *Amphimerus* infection and more reliable conclusions regarding its transmission dynamics. Furthermore, only one sample was obtained from each animal on one occasion. Although the samples in this study were concentrated to improve sensitivity, future study could involve taking multiple samples on different occasions with duplicate analyses to further improve diagnostic accuracy. On the other hand, surveyed animals were not examined for clinical symptoms, though some were presented sick and emaciated, with others looking healthy. Future research will examine infected animals in more depth and record any visible signs of ill-health.

Conclusions

This study is of importance in showing that the liver fluke *Amphimerus* sp. can infect and is common in cats and dogs living in Chachi communities of Ecuador, where human amphimeriasis is prevalent. The key finding of the study is that cats and dogs serve as definitive hosts and represent reservoirs for human infection. It can therefore be concluded that amphimeriasis is a zoonotic disease. These results provide relevant data that could be used for policy makers for conducting effective control strategies and measures against *Amphimerus* infection. As this study found a high prevalence of infection in cats and dogs, the recommended public health measure to control transmission would be to treat these domestic animals, as well as humans, with a specific drug for flukes such as praziquantel.

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Author Contributions

Conceived and designed the experiments: MC WC. Performed the experiments: MC WC RA MS AS HK HS. Analyzed the data: MC WC MS RA. Wrote the paper: MC WC MS RA.

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3.3 ARTÍCULO 3: Enzyme-linked immunosorbent assay for diagnosis of *Amphimerus* spp. liver fluke infection in humans.

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RESUMEN

El objetivo principal de este trabajo fue desarrollar, estandarizar y aplicar un ensayo de inmunoabsorción enzimática (ELISA) utilizando antígeno crudo de *Amphimerus* spp., para el diagnóstico serológico de la amphimeriosis. *Amphimerus* spp. es un parásito que puede potencialmente provocar enfermedad del hígado infectando a los seres humanos y animales domésticos. Es muy frecuente en algunas comunidades ecuatorianas en Sudamérica. Actualmente, el diagnóstico se basa en la observación microscópica directa de los huevos en las heces, pero con una baja sensibilidad de detección. Se necesitan métodos de diagnóstico más sensibles.

Los antígenos somáticos se obtuvieron de parásitos adultos de *Amphimerus* spp. Se analizaron 219 sueros humanos: 48 de individuos con infección confirmada por microscopía óptica con *Amphimerus* spp., 78 de ecuatorianos no infectados y que residen en zonas endémicas, 60 de personas no infectadas y que viven en zonas no endémicas (20 ecuatorianos, 20 europeos y 20 africanos) y 33 que tuvieron otras infecciones parasitarias y no parasitarias. Los resultados se analizaron utilizando el análisis mediante curvas ROC. El área bajo la curva fue de 0,967, indicando que la exactitud del ELISA fue alta. La sensibilidad fue del 85,0% [intervalo de confianza del 95% (IC): 80,3-89,7%] y la especificidad del 71,0% (IC del 95%: 65,2-76,8%). Se detectó reactividad cruzada contra *Paragonimus mexicanus, Fasciola hepatica*, *Schistosoma mansoni*, *Taenia solium*, *Strongyloides stercoralis*, *Mansonella* spp. y *Vampirolepis nana*.

Hemos desarrollado la primera técnica ELISA que detecta IgG anti-*Amphimerus* spp. en sueros humanos con buena sensibilidad, repetitividad y reproducibilidad. Sin embargo, se necesitan antígenos más específicos para mejorar el rendimiento de este ensayo. Esta prueba ELISA podría ser útil para el diagnóstico precoz y el tratamiento oportuno de infecciones por *Amphimerus* spp.

Enzyme-linked immunosorbent assay for diagnosis of *Amphimerus* spp. liver fluke infection in humans

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BACKGROUND *Amphimerus* spp. is a liver fluke that infects humans and domestic animals. It is highly prevalent in some Ecuadorian communities. Currently, diagnosis is based on the microscopic observation of eggs in faeces, but this has variable sensitivity. More sensitive methods are needed for diagnostic testing.

OBJECTIVE The main objective of this work was to develop an enzyme-linked immunosorbent assay (ELISA) using crude antigens from *Amphimerus* spp. adult worms to detect anti-*Amphimerus* IgG in human sera.

METHODS Crude somatic antigens were obtained from adult *Amphimerus* spp. worms. Human sera from 119 patients were tested: 48 from individuals with a confirmed *Amphimerus* spp. infection, 78 from non-infected Ecuadorians living in the endemic region, 60 from persons living in non-endemic areas (20 Ecuadorians, 20 Europeans, and 20 Africans), and 33 who had other parasitic and non-parasitic infections.

PRINCIPAL FINDINGS Results were analysed using the receiver-operator characteristic (ROC) curve analysis with an area under curve (AUC) value of 0.967. The accuracy of the ELISA was high. The sensitivity was 85.0% [95% confidence interval (CI): 80.3-89.7%] and the specificity was 71.0% (95% CI: 65.2-76.8%). Some cross reactivity was detected against *Paragonimus mexicanus*, *Fasciola hepatica*, Schistosomiasis, *Taenia solium*, *Strongyloides stercoralis*, *Mansonella* spp., and *Vampirolepis nana*.

MAIN CONCLUSIONS We have developed the first ELISA technique that detects anti-*Amphimerus* IgG in human sera with good sensitivity, repeatability and reproducibility. However, more specific antigens are needed to further enhance performance of this assay. Regardless, this ELISA test could be useful for early diagnosis and prompt treatment of human *Amphimerus* spp. infections.

Key words: *Amphimerus* spp. - Ecuador - ELISA - diagnosis

Amphimeriasis is a zoonotic disease caused by infection with the liver fluke *Amphimerus* spp., a member of the *Opisthorchiidae* family that includes *Clonorchis sinensis*, *Opisthorchis viverrini* and *O. felineus*. The genus *Amphimerus* (Barker, 1911) infects several wild and domestic mammals in the Americas, and it has been reported in cats, dogs, marsupials, and rodents from Canada, the United States, Costa Rica, Panama, Colombia, Ecuador, Brazil and Peru (Artigas & Perez 1962, Thatcher 1970, Miyazaki et al. 1978, de Moraes Neto et al. 1998, Bowman 2002). These flukes infect humans after ingestion of raw or undercooked freshwater fish parasitised with viable metacercariae. Recently, *Amphimerus* spp. infections were found in 34% of an indigenous Chachi population living in the tropical rain forest of Northwestern Ecuador. Since the Chachi community habitually consumes smoked or lightly cooked freshwater fish, an estimated 20,000 people are at risk of acquiring this disease (Calvopiña et al. 2011). Furthermore, a recent study reported a very high prevalence of infection in domestic cats and dogs living in Chachi communities (Calvopiña et al. 2015). Further studies by the authors (MC & WC) found infected people in several other provinces of Ecuador (unpublished observations).

Adult parasites of the genus *Amphimerus* spp. grow and parasitise the host's intra- and extra-hepatic bile ducts (Calvopiña et al. 2015). It is well documented that other members of the *Opisthorchiidae* are responsible for heavy and long-lasting infections that lead to hepatobiliary diseases including hepatomegaly, cholangitis, cholecystitis, and cholangiocarcinoma (Sripa et al. 2011). *Amphimerus* spp. infection may also occur in humans. However, it is mostly an asymptomatic disease, occasionally causing non-specific, generalised symptoms. To date, there are no comprehensive descriptions of this disease. However, histopathological studies in cats and a double-crested cormorant infected with *Amphimerus* spp. showed the presence of liver cirrhosis and pancreatitis (Rothenbacher & Lindquist 1963, Pense & Childs 1972).

At present, the diagnosis of *Amphimerus* spp. infections in humans is achieved by direct microscopic observation of eggs in the patient's faeces. Observation of eggs after formalin-ether concentration has also been utilised. The formalin-ether method is used on samples from cats

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and dogs, but this technique is not routinely conducted in local laboratories in Ecuador (Calvopiña et al. 2015). The sensitivity of direct microscopic observation is up to ten times lower than the formalin-ether method (Calvopiña et al. 2011). Likewise, it has been demonstrated that the fluke eggs of other *Opisthorchiidae* parasites can be detected in the stools. This represents the best way to obtain a definitive diagnosis, although this approach becomes increasingly unreliable in cases of low-worm burden (Johansen et al. 2010). Moreover, human amphimeriasis is asymptomatic in most cases and does not show pathognomonic signs and symptoms. Therefore, physicians can easily miss *Amphimerus* spp. infections or have difficulties making a differential diagnosis in endemic areas and, even more so, in non-endemic areas where infected migrant people may require medical attention. A reliable diagnosis test is needed to ensure appropriate treatment and prevent chronicity to reduce the risk of developing liver damage.

Immunological techniques, such as antibody-based methods using enzyme-linked immunosorbent assay (ELISA), have shown high sensitivity and specificity for diagnosing various parasitic infections (Elkins et al. 1991, Guevara et al. 1995). Of particular note, ELISA was used to detect parasites of the *Opisthorchiidae* family, and this technique performed the best among all serological tests evaluated (Meniavtseva et al. 1996). *Clonorchis sinensis* and *Opisthorchis* spp. induce robust immune responses and significantly increase the levels of IgG in experimental animals, which is similar to observations in humans (Elkins et al. 1991, Gómez-Morales et al. 2013). The detection of specific antibodies has been considered a complementary tool to establish a definitive diagnosis for liver fluke infections (Upatham & Viyanant 2003). Antigen-based techniques using crude adult extracts have been used for immunodiagnosis of *O. viverrini* and *C. sinensis* infections, albeit with varying levels of sensitivity and specificity (Wongratanacheewin et al. 1988, 2003, Poopyruchpong et al. 1990, Sawangsoda et al. 2012).

For *Amphimerus* infections, there are no immunological diagnostic methods currently available. In the present study, we developed an immunological assay using total crude somatic extract antigens to detect anti-*Amphimerus* IgG antibodies in sera from infected people in Ecuador.

MATERIALS AND METHODS

Ethics statement - The present study was approved by the Ethics Committee of the Universidad Central del Ecuador (License number LEC IORG 0001932, FWA 2482, IRB 2483, COBI-AMPHI-0064-11). Oral informed consent was obtained from all individuals participating in the study prior to the collection of biological samples for parasitological and immunological evaluation. Furthermore, written consent from animal owners was obtained prior to the euthanisation of cats that had been naturally infected with *Amphimerus* spp. to obtain adult parasites.

Crude somatic extract of Amphimerus spp. and antigen preparation - Adult *Amphimerus* spp. were obtained from cat livers following euthanasia by injection of a lethal dose of ketamine. The adult worms were washed with sterile phosphate-buffered saline (PBS) until the host's blood was completely removed. Afterwards, clean liver flukes were suspended in sterile PBS at a concentration of 30 worms/mL and homogenised in a buffer [10 mM Tris base, 1 mM EDTA, 500 µL of a protease inhibitor cocktail (UltraCruz® Protease Inhibitor Cocktail Tablet, Santa Cruz Biotechnology, catalogue number sc - 29130)]. The suspension was frozen and thawed three times and sonicated three times at 70 kHz for one minute each cycle using a sonicator (Vibra-Cell, Sonics and Materials Inc., Danbury, CT, USA). The samples were then centrifuged at 16000 *g* for 30 min at 4ºC. The crude antigen was lyophilised and stored at 4ºC until used. The protein concentration of the antigen was determined using the commercial micro-BCA™ Protein Assay Kit (Thermo Scientific Pierce, Waltham, MA, USA).

Human sera - A total of 219 human serum samples were used to test the diagnostic value of the crude antigen by ELISA. Of these, 48 sera samples were from patients who had received a definitive diagnosis, which was demonstrated microscopically by the presence of *Amphimerus* spp. eggs in their stools [true positive (TP)]. Additionally, 60 sera samples were from people living in non-endemic areas, including 20 Ecuadorians, 20 Europeans, and 20 Africans, who were free of trematode infections [true negative (TN)]. A group of 78 serum samples were obtained from people living in endemic areas, all of whom tested negative for a *Amphimerus* spp. infection based upon a coprologic test. Finally, 33 serum samples were derived from patients infected by other parasites or viruses including *Paragonimus mexicanus* (2), *Fasciola hepatica* (3), *Schistosoma mansoni* (2), *Schistosoma haematobium* (2), *Echinococcus granulosus* (2), *Mansonella* spp. (2), hookworms (2), *Vampirolepis nana* (2), *Strongyloides stercoralis* (2), *Taenia solium* (1), *Ascaris lumbricoides* (2), *Onchocerca volvulus* (1), *Leishmania* spp. (1), *Giardia intestinalis* (2), *Chilomastix mesnili* (1), *Blastocystis hominis* (1), *Microsporidium parvum* (1), *Plasmodium falciparum* (1), *Dientamoeba fragilis* (1), and hepatitis B virus (2). Of all the serum samples used in this study, 81% were from adults (14-98 years old), and approximately 45% were from females.

Serum samples from Europeans and Africans, as well as individuals with other parasitic and viral infections, were provided by IBSAL-CIETUS. The diagnoses of parasitic infections were based on parasitological and/or serological tests. Sera from Ecuadorians were obtained by venipuncture using disposable needles and 10 mL vacuum tubes (VACUETTE® Bio-one GmbH, Austria). These samples were obtained between January and May 2015. Blood was allowed to clot, and was subsequently centrifuged for 15 min at 1000 *g*. Serum was then aliquoted in cryovials and stored at -20ºC until use. All participants provided oral informed consent before blood samples were collected.

ELISA - A standard ELISA protocol was developed to test all human serum samples. Briefly, 96-well microtiter plates (Sigma, St. Louis, MO, USA) were coated with 100

µL/well of 4 µg/mL *Amphimerus* spp. crude extract antigens from adults. The crude extract had been reconstituted in carbonate buffer (pH 9.6). The plates were incubated overnight at 4ºC. Next, the plates were washed three times with PBS (pH 7.3) containing 0.05% Tween-20 (PBS-T20) (Sigma). Plates were then blocked in 200 µL/well of PBS with 0.5% BSA and 1% Tween-20 at 37ºC for 1 h. After additional washing steps, 100 µL of sera diluted 1:50 in PBS-T-20 was added to each well in duplicate, and plates were incubated at 37ºC for 1 h. After another wash step, 100 µL/well of 1:1000 diluted goat anti-human IgG peroxidase-labelled antibody (Sigma) was added and incubated at 37ºC for 1 h. Finally, another wash was performed and 100 µL/well of 5.3 µg/mL O-phenylene-diamine (OPD) and 8 µL of hydrogen peroxide in citric acid buffer were added. Plates were incubated at room temperature for 15 min, and the reaction was stopped by adding 50 µL/well of 1N H_2SO_4 solution. Optical density (OD) values were obtained by reading the plates at 492 nm using an ELISA plate microtitre reader (Ear400FT ELISA reader Lab. Instruments). All samples were tested in duplicate, and the OD mean was calculated.

Statistical analysis - A serological index (SI) was calculated for each OD, and this was used to establish a cut-off value for the ELISA using the following formula: SI = [(PS-NC) / (PC-NC)] X 100, where NC and PC are the negative and positive controls, respectively, and PS is the problem sample (Hernández-González et al. 2008).

Receiver-operator characteristic (ROC) curve analysis was used to determine the diagnostic value. The ROC curve is a graphical plot of the sensitivity versus "1 - specificity" for a binary classifier system. Using various cut-off values, this allows the selection of the cut-off value that gives the best balance of sensitivity and specificity for the test under consideration (Gómez-Morales et al. 2013). In order to determine which cut-off provided the most accurate result, we used the mean OD of the duplicates and the SI to calculate the area under the ROC curve for each cut-off. The specificity and sensitivity were interpreted according to the ROC analysis. The SI and OD in the different groups were expressed as the mean and standard error of the mean (SEM). Positive and negative predictive values were calculated, as described elsewhere (Fernández & Días 2003).

All statistical calculations were performed using SPSS (version 22.0), which is available from: https://www.ibm.com.

RESULTS

ROC curves were built with data from two defined true positive and true negative reference populations. The ODs of the 219 sera analysed are presented in Fig. 1. The mean OD ± standard deviation (SD) for the group of patients with confirmed amphimeriasis was the highest (0.938 ± 0.304) of those calculated. The remaining groups presented low means: ODs: 0.627 ± 0.144 for healthy patients from zones endemic for amphimeriasis, 0.412 ± 0.120 for healthy people from non-endemic areas, and 0.566 ± 0.206 for patients with other helminths, protozoan, and viral infections. Comparative analysis of the mean ODs detected significant differences between values for group 1 and those for the remaining groups ($p < 0.0001$). The area under curve was 0.967 for the mean OD value of the duplicates and 0.970 for the SI, indicating that the two parameters provided equally accurate results (Fig. 2). The ROC optimised cut-off was 18% for SI and 0.624 for the OD values; on the basis of these cut-off values, the sensitivity reached 85% (95% CI 78.3% to 91.7%) and the specificity was assessed as 94% (95% CI: 89.5% to 98.5%).

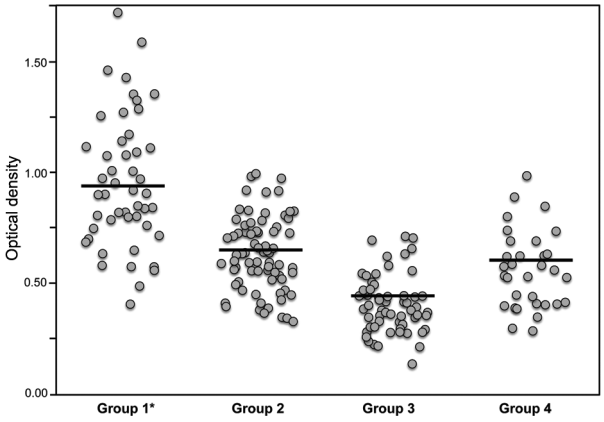

Fig. 1: values of optical density determined for the *Amphimerus* spp. crude antigen determined by enzyme-linked immunosorbent assay (ELISA) using sera from patients. We included sera from patients diagnosed with amphimeriasis based upon the detection of eggs (group 1), sera from healthy people living in amphimeriasis-affected areas (group 2), sera from healthy people living in areas unaffected by amphimeriasis (group 3), and sera from patients suffering from unrelated helminthic, protozoan, or viral infections (group 4). The horizontal line represents the mean values. The asterisk represents significant differences in the mean between group 1 and the other groups.

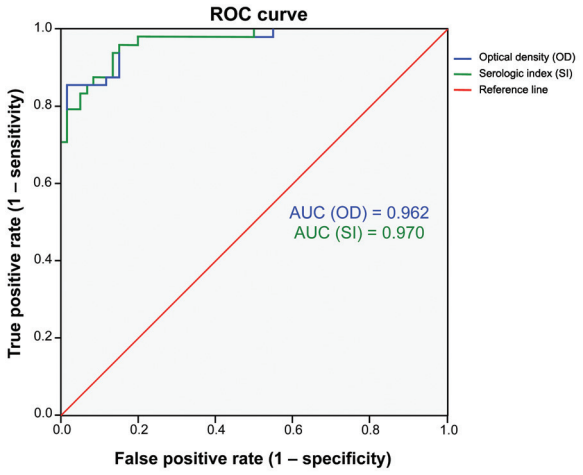

Fig. 2: the receiver-operator characteristic (ROC) curve. The ROC curve was generated using data from 48 sera samples from people infected with *Amphimerus* spp. and 60 sera samples from healthy people residing in non-endemic areas. The area under the curve (accuracy) for the serologic index (SI) was 0.970. For the optical density (OD) was 0.962.

TABLE

Diagnostic sensitivity, specificity, and positive and negative predictive values [cut-off 18%, 95% confidence interval (CI)] for serum samples from people diagnosed with *Amphimerus* spp. infection. The infection was confirmed by detection of *Amphimerus* eggs in stool samples (infected). Serum samples from people living in endemic areas who were negative for the presence of *Amphimerus* eggs in stool and serum samples from people suffering from other parasitic or non-parasitic infections (uninfected) were also examined

	Amphimerus spp. infection				
ELISA result	Infected	n	Uninfected	n	Total
Positive	TP	41	FP	48	89
Negative	FN	7	TN	123*	130
Total		48		171	219

Sensitivity: = 85.0% (95% CI: 80.3 - 89.7%), it was calculated as TP/(TP+FN); specificity: = 71.0% (95% CI: 65.2 - 76.8%), it was calculated as TN/(TN+FP); positive predictive value = 46.0% (95% CI: 39.4 - 52.6%), it was calculated as TP/(TP+FP); negative predictive value = 86.5 (95% CI: 84.2% to 88.8%), it was calculated as TN/(TN+FN); ELISA: enzyme-linked immunosorbent assay; FN: false negative; FP: false positive; TN: true negative; TP: true positive; *: for this value we eliminated 78 healthy people from endemic amphimeriasis area.

The diagnostic sensitivity and specificity of the ELISA in this study, including serum samples from people who lived in endemic areas and people with other parasitic and non-parasitic infections, are summarised in Table. The sensitivity of the test was 85% (95% CI: 80.3% to 89.7%), but the specificity dropped to 71% (95% CI: 65.2% to 76.8%) with a positive predictive value of 46% (95% CI: 39.4% to 52.6%) and negative predictive value of 86.5 (95% CI: 84.2% to 88.8%). For this reason, we eliminated 78 healthy people from areas where amphimeriasis is endemic.

Seven serum samples (17%) yielded false-negative results, and 48 samples (28%) yielded false-positive results. Of these false-positives, 34 were from people who live in endemic areas, and 14 were from persons with other parasitic infections (two were infected with *P. mexicanus*, three with *S. mansoni*, and one for each of these parasites: *T. solium*, *S. stercoralis*, *F. hepática*, *Mansonella* spp., and *V. nana*).

DISCUSSION

The present study shows for the first time that the *Amphimerus* spp. liver fluke infection induces an immune response in human hosts. This response is characterised by the production of IgG antibodies, which can be detected by ELISA using crude somatic extract antigens. Our data demonstrate that the ELISA has good sensitivity for sera from patients confirmed to be infected with this parasite.

Human amphimeriasis is an emerging food-borne trematode infection commonly found in tropical areas of Ecuador. It has a high prevalence in the indigenous Chachi population of Ecuador (Calvopiña et al. 2011). Our recent unpublished findings demonstrate that people in other communities along the Pacific coast of Ecuador are also infected with *Amphimerus* spp. In those studies, direct egg detection via faecal examination by microscopy was performed. However, as reported before, the formalin-ether concentration technique was more sensitive than a direct smear (Calvopiña et al. 2011). For *Opistorchiidae* family members, many of these trematodes have morphologically indistinguishable eggs. This includes minute intestinal trematodes, such as *Haplorchis* spp., *Echinostoma* spp., *Metorchis* spp., and *Metagonimus* spp. As a result, the low specificity of egg identification during common low-grade infections is a major problem for diagnostic methods (Johansen et al. 2010). Several studies for other *Opisthorchiidae* flukes such as *C. sinensis* and *O. viverrini*, have demonstrated that crude antigens work well for inducing a B-immune response during human infections (Wongratanacheewin et al. 1988, Meniavtseva et al. 1996). Thus, more sensitive and versatile serodiagnostic tests based on ELISAs have become the predominant assays used in biomedical research to diagnose clonorchiasis, opistorchiasis, paragonimiasis, and fasciolosis.

To the best of our knowledge, no serological tests have been developed and validated for amphimeriasis using crude antigens. This was our rational for evaluating ELISAs against a large panel of sera from individuals confirmed to have a *Amphimerus* spp. infection; people living in endemics area who are negative for eggs; people infected with viruses, protozoa, and other helminths; and, lastly, healthy people.

Regarding sensitivity, we found that our ELISA detected 85.0% (95% CI: 80.3-89.7%) of the TP samples. Our results are in accordance with those obtained for other parasites of the *Opisthorchiidae* family using crude antigens. For instance, the sensitivity of a previously reported ELISA for *C. sinensis* infection was 88.2% (Choi et al. 2003), and for *O. viverrini* infection, the range was between 83.3% and 100% (Poopyruchpong et al. 1990). This moderate sensitivity could be explained by level of worm burden, as the serum IgG levels against *O. viverrini* correlated with the overall egg count (Elkins et al. 1991, Wongratanacheewin et al. 2003). We propose that the sensitivity could also be affected by free-living parasites in the bile ducts.

In our results, 34 (19.8%) serum samples were not identified as positive for an infection by microscopic examination. However, these samples were correctly scored as positive sera by ELISA. This considerable improvement in the identification of seropositive cases for *Amphimerus* spp. in egg-negative individuals is probably due to the presence of a paucisymptomatic disease or a delayed diagnosis, which is common in *Opisthorchis* spp. infections (Armignacco et al. 2008). This suggests that this ELISA will be able to positively diagnose more infected people than faecal examination by microscopy can. In the ELISA, more than 95% of the differences between OD values of serum duplicates were less than two SDs, indicating the high reproducibility of the ELISA.

On the other hand, the specificity of the ELISA dropped to 71% (95% CI: 65.2% to 76.8%) when sera from people living in endemic areas or from those with

other health disorders unrelated to *Amphimerus* spp. were tested. Several authors have hypothesised that the main drawback of serologically based diagnostic methods that detect circulating antibodies, especially for infections caused by helminths, is the cross-reactivity that can occur when testing crude extracts containing parasites (Hong 1988, Sirisinha et al. 1990). This is particularly important in developing countries where people could be infected with several species of liver and intestinal flukes, in addition to other helminths (Sawangsoda et al. 2012). The relatively low specificity we observed was similar to results obtained in comparable studies of infections of *C. sinensis* and *Opisthorchis* spp. In South Korea, the ELISA for *C. sinensis* yielded an extremely low specificity of 33.3% (Kim et al. 2010). However, in another study, the specificity improved to 87.8% (38) and 81% for *O. viverrini* (Sripa et al. 2012).

Importantly, the specificity of our assay was assessed using heterologous sera from 33 patients infected with various other parasites. This included 23 helminths and eight protozoa, such as the liver fluke *F. hepatica*, the lung fluke *P. mexicanus*, and the blood flukes *S. haematobium* and *S. mansoni*. Sera from all of these pathogen-infected patients yielded false-positive results that impacted the overall specificity of the test, indicating that the use of somatic antigens in the diagnosis of amphimeriasis in areas where paragonimiasis is endemic produces many false-positive results. Of note, however, only paragonimiasis in Ecuador occurs in endemic areas where sera were collected (Calvopiña et al. 2014). Another important limitation of the current study is that we did not evaluate sera from patients infected with other *Opistorchiidae* family members, *C. sinensis* and *Opisthorchis* spp. In Ecuador, where amphimeriasis is currently prevalent, there are no reported cases of clonorchiais, opistorchiasis, or infections with another minute intestinal trematodes that occurs mainly in Asian countries (Johansen et al. 2010, Calvopiña et al. 2011). Thus, this ELISA method may be sufficient to accurately diagnose infections of *Amphimerus* spp. in endemic regions of Ecuador.

In summary, we have developed an ELISA that would be useful for epidemiological surveys in areas where *Amphimerus* spp. is endemic. However, further attempts to refine the assay are needed. This includes obtaining antigens that are more specific for *Amphimerus* spp., such as the excretory/secretory antigens that have provided more specificity than somatic soluble extracts for other trematode detection methods (Gómez-Morales et al. 2013). Regardless, our test shows good overall performance for the diagnosis of the *Amphimerus* infection as defined by sensitivity, repeatability, and reproducibility. We conclude that this method is useful for detecting anti-*Amphimerus* antibodies in human sera, facilitating early diagnosis and prompt treatment.

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AUTHORS' CONTRIBUTION

AG, WC, MC and AM - Designed the study, performed experiments, wrote the draft, and reviewed the manuscript; MC, VN and WC - did the fieldwork to collect *Amphimerus* spp. adult samples and Ecuadorian sera samples; AG and VN - prepared the crude *Amphimerus* spp. Antigen; BVS, JLA, PFS and WC - performed the ELISAs; WC, JLA and PFS - performed the statistical analysis and interpreted the results. All authors participated in drafting the article and approved the submitted version.

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3.4 ARTÍCULO 4: LAMPhimerus: a novel lamp assay for detecting *Amphimerus* spp. DNA in human stool samples.

William Cevallos, Pedro Fernández-Soto, Manuel Calvopiña, Cristina Fontecha-Cuenca, Hiromu Sugiyama, Megumi Sato, Julio López Abán, Belén Vicente, Antonio Muro.

PLoS Negl Trop Dis. 2017 Jun 19; 11(6):e0005672. doi: 10.1371.

RESUMEN

La amphimeriosis es una enfermedad transmitida por el consumo de peces de agua dulce y causada por el trematodo hepático *Amphimerus* spp. Recientemente ha sido reportada como endémica y con alta prevalencia en humanos y animales domésticos que viven en la costa pacífica de Ecuador. El diagnóstico se basa en el examen de heces para identificar los huevos del parásito, pero carece de buena sensibilidad. Además, la morfología de los huevos puede confundirse con otros trematodos hepáticos e intestinales. Hasta la fecha no se han desarrollado métodos inmunológicos o moleculares. Se requiere de nuevas técnicas de diagnóstico en muestras clínicas para la detección sensible y específica de ADN de *Amphimerus* spp.

Se diseñó el método LAMP dirigido a una secuencia de la región de transcripción interna (ITS2) de *Amphimerus* spp. Para optimizar los ensayos moleculares se obtuvo ADN de parásitos adultos recuperados de animales. La PCR convencional se realizó usando los cebadores externos F3-B3 para verificar la correcta amplificación de la secuencia de ADN diana de *Amphimerus* spp. La técnica LAMP se optimizó utilizando diferentes mezclas de reacción y temperaturas. Finalmente se estableció la más apropiada como LAMPhimerus. Se evaluó la especificidad y sensibilidad de la PCR y LAMP. El límite de detección fue de 1 pg de ADN genómico. Las pruebas de campo se realizaron utilizando 44 muestras de heces humanas recolectadas en localidades donde esta trematodosis es endémica. Veinticinco muestras fueron microscópicamente positivas para la detección de huevos de *Amphimerus* spp. En las pruebas moleculares, la PCR (F3-B3) resultó negativa cuando se analizó el ADN de muestras fecales. Cuando se analizaron todas las muestras de heces humanas incluidas en nuestro estudio, la sensibilidad y especificidad diagnósticas para nuestro ensayo LAMPhimerus, fueron 76,67% y 80,77%, respectivamente.

En conclusión, hemos desarrollado y evaluado, por primera vez, un ensayo LAMP específico y sensible para detectar *Amphimerus* spp. en muestras de heces humanas. El procedimiento ha sido denominado LAMPhimerus y tiene el potencial de ser adaptado para el diagnóstico de campo y la vigilancia de la amphimeriosis en áreas endémicas. Futuros estudios a gran escala evaluarán la aplicabilidad de este novedoso ensayo LAMP.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

LAMPhimerus: A novel LAMP assay for detecting *Amphimerus* sp. DNA in human stool samples

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Abstract

Background

Amphimeriasis is a fish-borne disease caused by the liver fluke *Amphimerus* spp. that has recently been reported as endemic in the tropical Pacific side of Ecuador with a high prevalence in humans and domestic animals. The diagnosis is based on the stool examination to identify parasite eggs, but it lacks sensitivity. Additionally, the morphology of the eggs may be confounded with other liver and intestinal flukes. No immunological or molecular methods have been developed to date. New diagnostic techniques for specific and sensitive detection of *Amphimerus* spp. DNA in clinical samples are needed.

Methodology/Principal findings

A LAMP targeting a sequence of the *Amphimerus* sp. internal transcribed spacer 2 region was designed. *Amphimerus* sp. DNA was obtained from adult worms recovered from animals and used to optimize the molecular assays. Conventional PCR was performed using outer primers F3-B3 to verify the proper amplification of the *Amphimerus* sp. DNA target sequence. LAMP was optimized using different reaction mixtures and temperatures, and it was finally set up as LAMPhimerus. The specificity and sensitivity of both PCR and LAMP were evaluated. The detection limit was 1 pg of genomic DNA. Field testing was done using 44 human stool samples collected from localities where fluke is endemic. Twenty-five samples were microscopy positive for *Amphimerus* sp. eggs detection. In molecular testing, PCR F3-B3 was ineffective when DNA from fecal samples was used. When testing all human stool samples included in our study, the diagnostic parameters for the sensitivity and specificity were calculated for our LAMPhimerus assay, which were 76.67% and 80.77%, respectively.

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Conclusions/Significance

We have developed and evaluated, for the first time, a specific and sensitive LAMP assay for detecting *Amphimerus* sp. in human stool samples. The procedure has been named LAMPhimerus method and has the potential to be adapted for field diagnosis and disease surveillance in amphimeriasis-endemic areas. Future large-scale studies will assess the applicability of this novel LAMP assay.

Author summary

Amphimeriasis, a fish-borne zoonotic disease caused by the liver fluke *Amphimerus* spp., is a highly prevalent parasitic infection affecting an indigenous Amerindian group, the Chachi, living in rural and remote tropical areas along the Río Cayapas and its tributaries in the north-western coastal rainforest of Ecuador. Very little is known about the clinical course and treatment of this disease, and the only method for diagnosing it is the parasitological microscopic detection of eggs from *Amphimerus* spp. in patients' stool samples. This method lacks sensitivity, and the morphology of the eggs may be confounded with other liver and intestinal flukes. New diagnostic tools that can improve the sensitivity and specificity for diagnosing *Amphimerus* spp. infection would be desirable. At present, LAMP technology shows all the characteristics required of a real-time assay with simple operation for potential use in the clinical diagnosis of infectious diseases, particularly in the field conditions in developing countries for most neglected tropical diseases. In this study, we developed and successfully evaluated a LAMP assay for detecting *Amphimerus* sp. in human stool samples. After further validation, our LAMP assay (LAMPhimerus) could be readily adapted for effective field diagnosis and disease surveillance in amphimeriasis-endemic areas.

Introduction

Amphimerus spp. are digenean parasitic flatworms in the bile ducts of birds, reptiles and mammals, and they are closely related to the genera *Clonorchis* and *Opisthorchis* within the Opisthorchiidae family [1, 2]. As for other members of the Opisthorchiidae family, the life cycle of *Amphimerus* spp. is highly complex, involving both freshwater snails and fish as intermediate hosts and vertebrates, including humans, as definitive hosts [3]. Humans or fish-eating animals are infected with *Amphimerus* spp. through the ingestion of raw or undercooked freshwater fish containing metacercariae [3]. Recently, *Amphimerus* sp. has been reported, for the first time, as endemic in rural communities in the tropical Pacific side of Ecuador with a high prevalence in humans and domestic cats and dogs, causing amphimeriasis [3, 4]. Several foodborne trematodiases around the world are now considered by the World Health Organization as neglected tropical diseases (NTDs) [5] with high prevalence, especially in East Asia [6], and they have serious consequences, such as cholangiocarcinoma [7,8]. Amphimeriasis has been reported as a new emerging foodborne zoonotic disease [3].

Amphimerus spp. adult stages are located in the bile ducts of the definitive host, and the eggs are shed in the feces [3]. Diagnosis of human and animal infection can be performed with the wet mount technique for examining feces, allowing for microscopic visualization of parasite eggs; the formalin-ether concentration method has been shown to increase the sensitivity

ten-fold [3]. Detection of the eggs in bile or duodenal fluid can also be performed. However, microscopic examination is cumbersome and time consuming, and it could have a low sensitivity in cases of light infections. In addition, the morphological similarity of the *Amphimerus* spp. eggs to those of closely related species belonging to genera *Clonorchis* and *Opisthorchis* as well as to minute intestinal flukes, makes diagnosis difficult. It would be necessary to use scanning electron microscopy to accurately observe the differences between the coatings of the different species [3]. Therefore, the development of a new method that can improve the sensitivity and specificity for diagnosing *Amphimerus* spp. infection is urgently required.

To overcome these limitations, the use of molecular approaches has become a powerful tool for the diagnosis, identification and differentiation of closely related species. In recent years, several polymerase chain reaction (PCR)-based molecular diagnostic methods have been developed for detecting many parasitic trematodes, including those species that are closely related to *Amphimerus* spp., such as *C. sinensis* [9–14] and *O. viverrini* [15–18]. Although these studies have demonstrated that PCR-based methods are very sensitive and specific, they are not still widely used in low-income countries because well-trained personnel and expensive equipment are needed, making them unviable for routine application in field conditions in endemic areas that are generally undeveloped and have a high disease prevalence. Loop-mediated isothermal amplification (LAMP) could be a good alternative amplification technology [19] because it has several salient advantages over most PCR-based methods [20, 21]. At present, LAMP technology has all the characteristics required of a real-time assay along with simple operation for potential use in the clinical diagnosis of infectious diseases, particularly under the field conditions in developing countries [22, 23]. Additionally, several LAMP assays have already been successfully described for detecting trematode parasites, including a number of species causing foodborne trematodiases, such as *Fasciola* spp. [24], *Clonorchis sinensis* [25, 26], *Opisthorchis viverrini* [27–29] and *Paragonimus westermani* [30].

With the aim of developing new, applicable and cost-effective molecular tools for the diagnosis of amphimeriasis, we have developed and evaluated, for the first time, a LAMP assay for the specific detection of *Amphimerus* sp. liver fluke in human stool samples.

Methods

Ethics statement

The study protocol was approved by the Ethics Committee of Universidad Central del Ecuador (License number: LEC IORG 0001932, FWA 2482, IRB 2483. COBI-AMPHI-0064-11) and the Ethics Committee of the University of Salamanca (protocol approval number 48531). Participants were given detailed explanations about the aims, procedures and possible benefits of the study. Written informed consent was obtained from all subjects prior to the collection of biological samples for parasitological and molecular evaluation. Parents or guardians of children who participated in the study provided written informed consent on the child's behalf. All samples were coded and treated anonymously.

Study area and population

The study was conducted during February 2016 in two indigenous Chachi villages alongside the Cayapas River in the Esmeraldas province, located in the northwest coastal rainforest of Ecuador [4]. The indigenous Chachi, living together with the Afro-ecuadorian and mestizo populations, belong to the predominant autochthonous group in this area, representing 13% of the inhabitants in this region. These communities are the same as those studied previously and have a high prevalence of infection (15.5% to 34.1%) with *Amphimerus* sp. Prevalences are also high in local cats and dogs [3, 4]. They live in remote villages where the only way to reach

them is by boat along the river. Sanitation facilities are lacking, and the members are hunters who habitually eat undercooked freshwater fish (mainly smoked fish) caught in the neighboring rivers [4]. More details on the region can be accessed elsewhere [31, 32].

Human stool samples and parasitological tests

Human stool samples were obtained from indigenous Chachi communities during February 2016. Each participant who enrolled in the study was given a copro-parasitological flask for stool collection. Samples were collected within a few hours of stool passing. After collection, samples were transported to the Parasitology Laboratory (Centro de Biomedicina, Universidad Central del Ecuador, Quito, Ecuador) for parasitological screening under light microscopy by direct examination, simple sedimentation, formalin-ether concentration and Kato-Katz techniques. All samples were examined by two qualified laboratory technicians according to the basic laboratory methods in medical parasitology recommended by the World Health Organization (WHO) [33]. After parasitological screening, a total of 44 stool samples were selected, including 25 (56.81%) that were positive for *Amphimerus* sp. eggs-by one or more parasitological methods-and 19 (43.18%) negative samples. Afterwards, the 44 stool samples that were well-preserved in 80% ethanol were sent to the Research Center for Tropical Diseases (CIETUS) at the University of Salamanca, Spain, for further DNA extraction and molecular analysis as described below.

DNA extraction for molecular analyses

DNA from human fecal samples. Approximately 250–300 mg from each of 44 stool samples preserved in 80% ethanol solution was used for DNA extraction. First, excess ethanol was removed from each vial; subsequently, DNA extraction was performed using the Mini Stool DNA Extraction kit (Macharey-Nagel) according to the manufacturers' instructions. Purified DNA samples were stored at -20˚C until use.

DNA from parasites. *Amphimerus* sp. genomic DNA was extracted from frozen adult worms that were previously obtained from the livers of naturally infected cats and dogs of Chachi communities, as described elsewhere [4], using a G-spin Total DNA Extraction Kit (Intron Biotechnology) according to the manufacturers' instructions. DNA was measured using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies) and then diluted with ultrapure distilled water to final concentrations of 5 ng/μL and 0.5 ng/μL. Serial 10-fold dilutions from adult *Amphimerus* sp. DNA were prepared with ultrapure water, ranging from $1x10^{-1}$ to $1x10^{-9}$, and stored at -20˚C until use. DNA thus prepared was used as a positive control in all PCR and LAMP reactions as well as for assessing the sensitivity of both molecular assays.

To determine the specificity of PCR and LAMP assays to amplify only *Amphimerus* sp. DNA, a total of 16 DNA samples from several helminths, including trematodes (*Clonorchis sinensis*, *Opisthorchis viverrini*, *Fasciola hepatica*, *Dicrocoelium dendriticum*, *Schistosoma mansoni*, *S. haematobium*, *S. japonicum*, and *S. intercalatum*), cestodes (*Echinococcus granulosus* and *Taenia truncata*), nematodes (*Onchocerca volvulus*, *Strongyloides venezuelensis*, and *Trichinella spiralis*) and protozoa (*Entamoeba histolytica*, *Cryptosporidium parvum*, and *Giardia duodenalis*) were used. The concentration of all DNA samples was measured by the same method as described for *Amphimerus* sp. DNA, which was then diluted with ultrapure water to a final concentration of 0.5 ng/μL and kept at -20˚C until use in molecular assays.

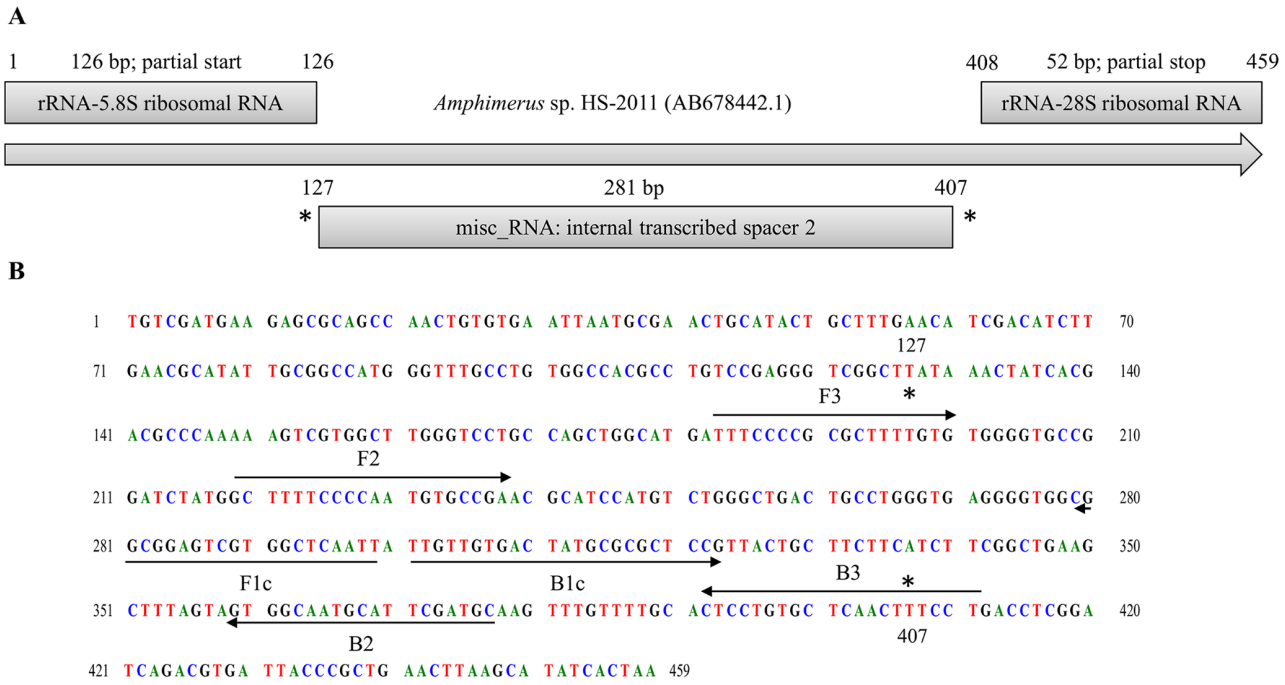
Designing LAMP primers

An 459 base pair (bp) sequence, corresponding to a linear genomic DNA partial sequence in the ITS2 region of *Amphimerus* sp. HS-2011 isolated from human host, was selected and

retrieved from GenBank (Accession No. AB678442.1) [4] for the design of the specific primers. The 459 bp sequence was tested using BLASTN analysis [34] for similarity in the available online genome databases. A set of LAMP primers complementary to the nucleotide sequence was designed using the online Primer Explorer V4 software (https://primerexplorer.jp/elamp4.0.0/; Eiken Chemical Co., Ltd., Tokyo, Japan) according to criteria described by Notomi et al [19]. A final complete set of four primers-including a forward outer primer (F3), a reverse outer primer (B3), a forward inner primer (FIP) and a backward inner primer (BIP)-was selected based on the criteria described in "A guide to LAMP primer designing" (http://primerexplorer.jp/e/v4_manual/index.html) of LAMP primers; the locations and target sequence are shown in Fig 1. All the primers were of HPLC grade (Thermo Fisher Scientific Inc., Madrid, Spain). The lyophilized primers were resuspended in ultrapure water to a final concentration of 100 pmol/μL and stored at -20˚C until use.

PCR using outer primers F3 and B3

The outer LAMP primer pair (F3 and B3; Fig 1) was initially tested for *Amphimerus* sp. specificity by a PCR to verify whether the correct target was amplified. PCR was conducted in 25 μL



C

Primer	Length (bp)	Sequence (5′-3′)
F3	18	TTTCCCCGCGCTTTTGTG
B3	20	AGGAAAGTTGAGCACAGGAG
FIP (F1c+F2)	39	AATTGAGCCACGACTCCGCCCTTTTCCCCAATGTGCCGA
BIP (B1c+B2)	41	TTGTTGTGACTATGCGCGCTCCGCATCGAATGCATTGCCAC

Fig 1. Design of LAMP primers for detecting DNA of *Amphimerus* sp. (A) Schematic representation of the 459 bp selected sequence of *Amphimerus* sp. HS-2011 isolated from human host (AB678442.1). (B) Location of the LAMP primers within the selected sequence. Arrows indicate the direction of extension. (C) Sequences of LAMP primers. F3, forward outer primer; B3, reverse outer primer; FIP, forward inner primer (F1c and F2 sequences); and BIP, reverse inner primer (B1c and B2 sequences).

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of a reaction mixture containing 2.5 μL of 10x buffer, 1.5 μL of 25 mmol/L $MgCl_2$, 2.5 μL of 2.5 mmol/L dNTPs, 0.5 μL of 100 pmol/L F3 and B3, 2 U *Taq*-polymerase and 2 μL (10 ng) of DNA template. Initial denaturation was conducted at 94˚C for 1 min, which was followed by a touchdown program for 15 cycles with successive annealing temperature decrements of 1.0˚C every 2 cycles. For these 2 cycles, the reaction was denatured at 94˚C for 20 s followed by annealing at 64˚C-58˚C for 20 s and polymerization at 72˚C for 30 s. The subsequent 15 cycles of amplification were similar, except that the annealing temperature was 57˚C. The final extension was performed at 72˚C for 10 min. All PCR reactions were performed in a Mastercycler Gradient-96well (Eppendorf).

The specificity of PCR F3-B3 was tested using heterogeneous DNA samples from other parasites included in the study. The sensitivity was also assayed to establish the detection limit of *Amphimerus* sp. DNA with 10-fold serial dilutions prepared as mentioned above. All PCR assays were performed with 2 μL of the DNA template (5 ng/μL) in each case. Negative controls (ultrapure water) and positive controls (genomic DNA from *Amphimerus* sp.) were always included. The PCR products (3–5 μL/each) were subjected to 1.5–2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV light.

Establishing the LAMP assay

We evaluated the LAMP primer set designed by using different reaction mixtures to compare results in *Amphimerus* sp. DNA amplification. LAMP reactions mixtures (25 μL) contained 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, 1.4 mM each of dNTP (Intron), 1x Isothermal Amplification Buffer-20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20 (New England Biolabs, UK)-betaine (0.8, 1, 1.2, 1.4 or 1.6 M) (Sigma, USA), supplementary $MgSO_4$ (2, 4, 6 or 8 mM) (New England Biolabs, UK) and 8 U of *Bst* polymerase 2.0 WarmStart (New England Biolabs, UK) with 2 μL (1 ng) of template DNA.

LAMP reactions were performed in 0.5-mL micro-centrifuge tubes that were incubated in a simple heating block at a range of temperatures (61, 63 and 65˚C) for 60 min to optimize the reaction conditions and then heated at 80˚C for 5–10 min to terminate the reaction. The optimal temperature was determined and used in the following tests. Because of the high sensitivity of the LAMP reaction, DNA contaminations were prevented using sterile tools at all times, performing each step of the analysis in separate work areas and minimizing manipulation of the reaction tubes. Template DNA was replaced by ultrapure water as a negative control in each LAMP reaction.

The specificity of the LAMP assay to amplify only *Amphimerus* sp. DNA was tested against 16 DNA samples obtained from other parasites used as heterogeneous controls, as mentioned above. To determine the lower detection limit of the LAMP assay, genomic DNA from *Amphimerus* sp., 10-fold serial diluted as mentioned above, was subjected to amplification compared with the PCR F3-B3.

Detection of LAMP products

The LAMP amplification results could be visually inspected by adding 2 μL of 1:10 diluted 10,000X concentration fluorescent dye SYBR Green I (Invitrogen) to the reaction tubes. Green fluorescence was clearly observed in the successful LAMP reaction, while it remained original orange in the negative reaction. In addition, the LAMP products (3–5 μL) were monitored using 1.5–2% agarose gel electrophoresis stained with ethidium bromide, visualized under UV light and then photographed using an ultraviolet Gel documentation system (UVItec, UK).

Statistical analysis

To estimate the accuracy of the LAMP assay method as a diagnostic test, the percentages of the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using the MedCalc statistical program version 16.8.4 (MedCalc Software, Ostende, Belgium) according to the software instruction manual (www.medcalc.org).

Results

PCR using outer primers F3-B3: Sensitivity and specificity

The expected 229 bp PCR product was successfully obtained with outer primers F3 and B3 from *Amphimerus* sp. DNA. According to sensitivity, the minimum level of *Amphimerus* sp. DNA detectable by PCR was 0.001 ng (1 pg) (Fig 2A). Additionally, when DNA samples from other parasites included in the study were subjected to this PCR assay, no amplicons were obtained (Fig 2B).

Examination of human stool samples by PCR F3-B3

We tested the 44 human stool samples by PCR using the outer primers F3 and B3, and very faint bands of the expected size (229 bp) were only obtained in 3 samples (nos. 31, 34 and 45) (S1 Fig).

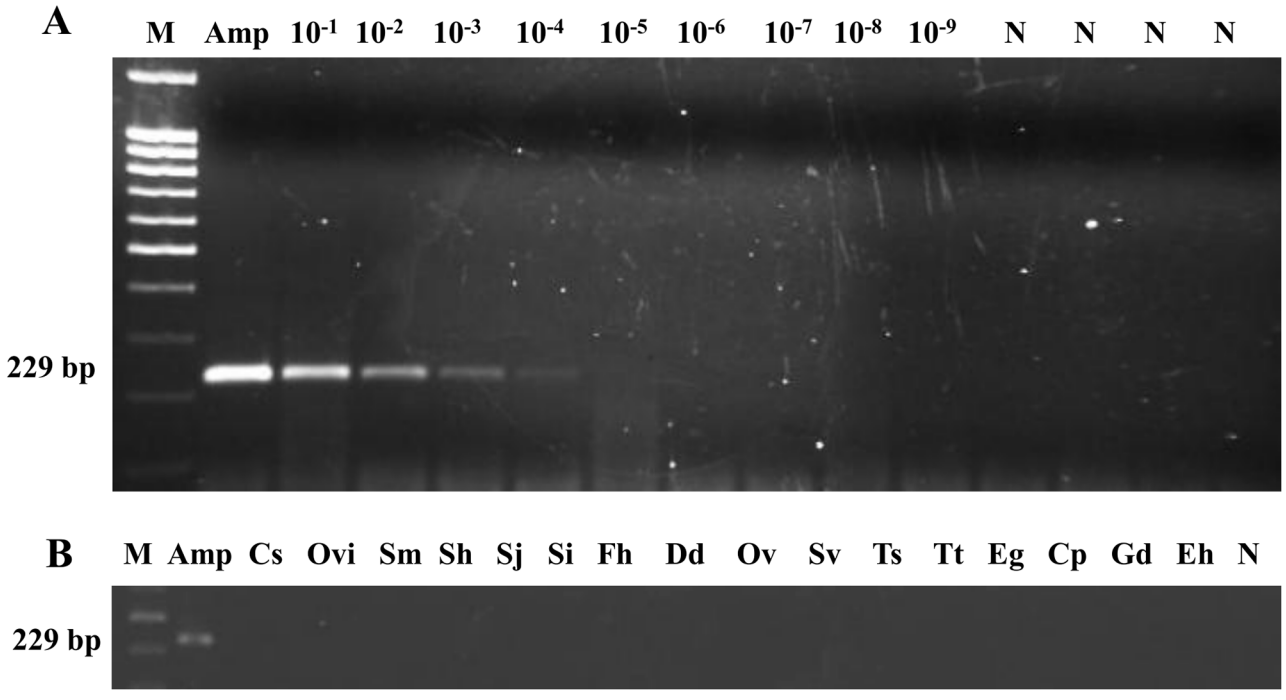

Fig 2. PCR verification, detection limit and specificity using outer primers F3 and B3 for DNA amplification of *Amphimerus* sp. (A) Detection limit of PCR F3-B3. Lane Amp, DNA of *Amphimerus* sp. (10 ng); lanes 10^{-1}–10^{-9}, 10-fold serial dilutions of *Amphimerus* sp. DNA. (B) Specificity PCR F3-B3. Lanes Amp, Cs, Ovi, Sm, Sh, Sj, Si, Fh, Dd, Ov, Sv, Ts, Tt, Eg, Cp, Gd, Eh: DNA samples of *Amphimerus* sp., *Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, *Fasciola hepatica*, *Dicrocoelium dendriticum*, *Onchocerca volvulus*, *Strongyloides venezuelensis*, *Trichinella spiralis*, *Taenia truncata*, *Echinococcus granulosus*, *Cryptosporidium parvum*, *Giardia duodenalis* and *Entamoeba histolytica*, respectively. In all panels: lane M, molecular weight marker (100 bp Plus Blue DNA Ladder) and lane N, negative controls (ultrapure water, no DNA template).

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Establishing the LAMP assay: LAMPhimerus

Subsequent to testing different reaction mixtures and temperature conditions, the best amplification results (based on the most evident color change by adding the fluorescent dye and the intensity of the multiple bands on agarose as well as reproducibility of tests) were always obtained when the LAMP master mixture contained 1 M betaine combined with supplementary 6 mM $MgSO_4$ (resulting in a final concentration of 8 mM $MgSO_4$ in 1x Isothermal Amplification Buffer) and was incubated at 63˚C for 60 min in a heating block (Fig 3A). When we evaluated the sensitivity of the established LAMP assay, the limit of detection in *Amphimerus*

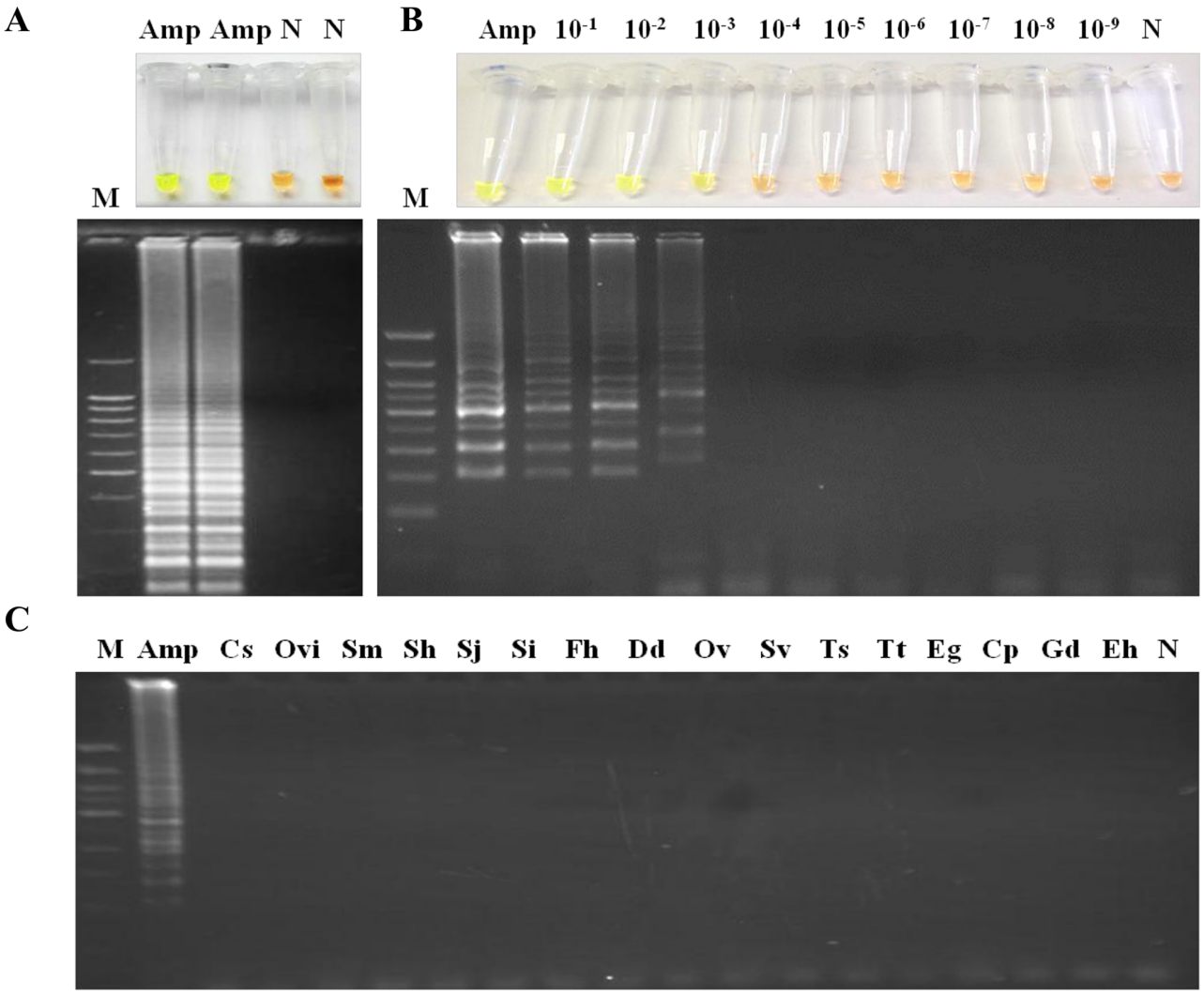

Fig 3. Establishing the LAMP assay. (A) LAMP amplification results obtained using the established LAMPhimerus assay with the addition of SYBR Green I (up) or visualization on agarose gel (down). Lane M, molecular weight marker (100 bp Plus Blue DNA Ladder); lane Amp, *Amphimerus* sp. DNA (1 ng); and lane N, negative control (ultrapure water and no DNA template). (B) Sensitivity assessment of LAMPhimerus using serial dilutions of *Amphimerus* sp. genomic DNA. Lane M, molecular weight marker (100 bp Plus Blue DNA Ladder); lane Amp, *Amphimerus* sp. DNA (1 ng); lanes 10^{-1}–10^{-9}, 10-fold serial dilutions; and lane N, negative control (ultrapure water and no DNA template). (C) Specificity of the LAMPhimerus assay. Lane M, molecular weight marker (100 bp Plus Blue DNA Ladder); lane Amp, Cs, Ovi, Sm, Sh, Sj, Si, Fh, Dd, Ov, Sv, Ts, Tt, Eg, Cp, Gd, and Eh: DNA samples of *Amphimerus* spp., *Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, *Fasciola hepatica*, *Dicrocoelium dendriticum*, *Onchocerca volvulus*, *Strongyloides venezuelensis*, *Trichinella spiralis*, *Taenia truncata*, *Echinococcus granulosus*, *Cryptosporidium parvum*, *Giardia duodenalis* and *Entamoeba histolytica*, respectively; and lane N, negative control (ultrapure water and no DNA template).

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sp. genomic DNA amplification was identical to that obtained when using PCR with outer primers, specifically 0.001 ng (1 pg) (Fig 3B). To determine the specificity of the LAMP assay for *Amphimerus* sp., a panel of 16 additional DNA samples from other parasites was tested for amplification. A positive result was only obtained when *Amphimerus* sp. DNA was used as template, while DNA samples from other specimens were not amplified, demonstrating its high specificity (Fig 3C).

In this way, the best reaction mixture, in addition to the specific primers designed, was established as the most fitting assay for amplification of *Amphimerus* sp. DNA and was named "LAMPhimerus" in all successive LAMP reactions.

Application of LAMP in human stool samples: LAMPhimerus analysis

The 44 human stool samples were tested with LAMPhimerus assay using two incubation times for reaction, 60 min and 120 min (Fig 4). To prevent potential cross-contamination, amplification assays were performed in four batches of 11 samples each for easy handling. When testing stool samples using an incubation time of 60 min (Fig 4A), we obtained LAMP positive results in 14/44 (31.81%) samples, including 5 samples (nos. 36, 45, 47, 68 and 99) that were negative in all parasitological tests applied. When using an incubation time of 120 min (Fig 4B), the number of positive results was increased to 22/44 (50%), which also included 4/5 negative parasitological samples as before (nos. 36, 45, 68 and 99). In all LAMP positive reactions, green fluorescence was clearly visualized under natural light conditions. Positive controls always worked well and negative controls were never amplified. All positive results obtained when performing the assay for 60 min were supported at 120 min, except one sample (no. 47). For 120 min, in sample no. 47, a mix between green and orange was observed in the reaction tube; also a very faint smear was visible on agarose gel, indicating poor DNA amplification. Taking together the results obtained from the two incubation assays, we finally considered sample no. 47 as positive, resulting in a total of 23/44 (52.27%) positive LAMPhimerus results.

In summary, of the total of 25 parasitologically positive stool samples, we obtained 9/25 (36%) and 18/25 (72%) positive results when we applied LAMPhimerus for 60 min and 120 min, respectively. Additionally, positive results included 5/19 (26.31%) samples (nos. 36, 45, 47, 68 and 99) that were negative in all previously applied parasitological tests. Of the 11 samples (nos. 6, 27, 30, 32, 33, 42, 54, 60, 79, 84, and 85) that were simultaneously positive on three parasitological tests (including the formalin-ether concentration technique, FECT; simple sedimentation technique, SST; and Kato-Katz technique, KKT), 9 (9/11; 82%) were also positive on the LAMP assay; only the 2 samples (nos. 42 and 54) with the same very low egg count (FEC = 1; EPG = 24) were negative on the LAMP assay. Fig 5 shows a comparison of the results obtained for detecting *Amphimerus* sp. in human stool samples when using the classical parasitological techniques applied and the 120 min-LAMPhimerus assay.

Considering the results obtained, the following diagnostic parameters for the sensitivity and specificity were calculated for our LAMPhimerus assay: 76.67% sensitivity (95% CI: 52.72% -90.07%); 80.77% specificity (95% CI: 60.65% -93.45%); 82.14% positive predicted value (95% CI: 67.13% -91.20%) and 75.00% negative predicted value (95% CI: 60.43% -85.49%).

Discussion

Human amphimeriasis, caused by the *Amphimerus* spp. liver fluke, has been recently reported as an emerging zoonotic food-borne trematodiasis [3, 4]. The conventional diagnosis of liver fluke infections in humans is based on the demonstration of eggs in different clinical samples, especially in feces. However, the morphological identification of eggs is troublesome in

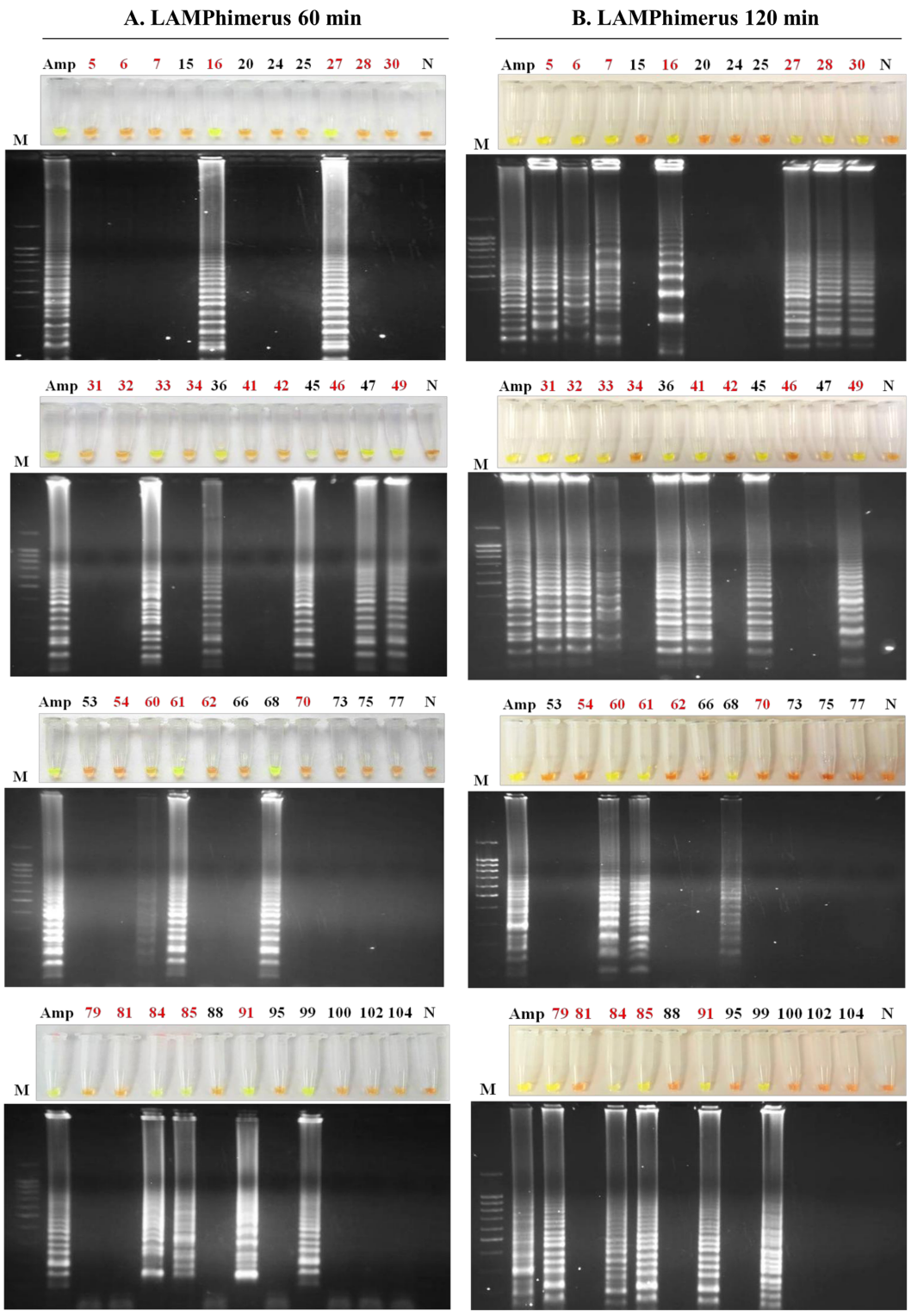

Fig 4. LAMPhimerus analysis of human stool samples in this study. (A) Incubation time of 60 min. (B) Incubation time of 120 min. Lane M, molecular weight marker (100 bp Plus Blue DNA Ladder); lane Amp, *Amphimerus* sp. DNA (1 ng); lane N, negative controls (ultrapure water and no DNA template); and numbers 5–104, analyzed human stool samples. The highlighted red numbers correspond to samples that were positive by one or more applied parasitological methods.

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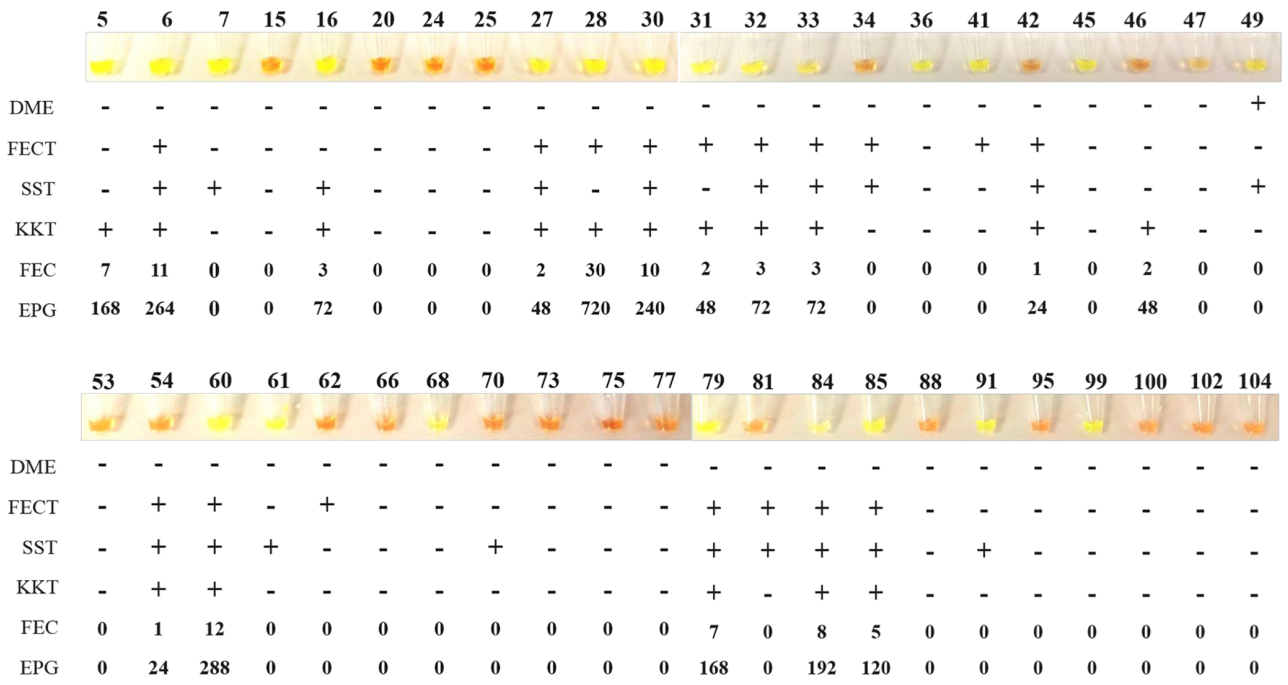

	5	6	7	15	16	20	24	25	27	28	30	31	32	33	34	36	41	42	45	46	47	49
DME	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
FECT	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	-	-	-	-
SST	-	+	+	-	+	-	-	-	+	-	+	-	+	+	+	-	-	+	-	-	-	+
KKT	+	+	-	-	+	-	-	-	+	+	+	+	+	+	-	-	-	+	-	+	-	-
FEC	7	11	0	0	3	0	0	0	2	30	10	2	3	3	0	0	0	1	0	2	0	0
EPG	168	264	0	0	72	0	0	0	48	720	240	48	72	72	0	0	0	24	0	48	0	0

	53	54	60	61	62	66	68	70	73	75	77	79	81	84	85	88	91	95	99	100	102	104
DME	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FECT	-	+	+	-	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-
SST	-	+	+	+	-	-	-	+	-	-	-	+	+	+	+	-	+	-	-	-	-	-
KKT	-	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-
FEC	0	1	12	0	0	0	0	0	0	0	0	7	0	8	5	0	0	0	0	0	0	0
EPG	0	24	288	0	0	0	0	0	0	0	0	168	0	192	120	0	0	0	0	0	0	0

Fig 5. Comparison of the results obtained by the LAMPhimerus assay and classical parasitological techniques applied in this study for detecting *Amphimerus* sp. in human stool samples. DME, direct microscopy detection; FECT, formalin-ether concentration technique; SST, simple sedimentation technique; KKT, Kato-Katz technique; FEC, fecal egg count; EPG, eggs per gram of feces; -, negative for egg detection; and +, positive for egg detection. Values indicated for FEC and EPG correspond to the numbers of detected eggs and numbers 5–104 correspond to the analyzed human stool samples.

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endemic areas where co-infection with other zoonotic trematodes usually exists. Additionally, stool examination lacks analytical sensitivity, particularly for light infections, requiring serial fecal sampling and an intensive effort in resource-poor settings [35]. To solve these limitations, many immunological and molecular diagnostic approaches have already been developed and applied to detect the presence of several human zoonotic trematode infections with varying accuracy [36, 37].

For detecting *Amphimerus* spp. infection, conventional coprological techniques are the only ones available, and no immunological or molecular methods have been developed to date. Among the possible molecular methods to be developed, LAMP tests are rapidly becoming an attractive diagnostic option for use under field conditions in laboratories with basic facilities [22, 23]. Hence, in this study, with the aim of improving the diagnostic testing for amphimeriasis, we have developed and evaluated, for the first time, a specific LAMP assay to detect *Amphimerus* sp. DNA in field samples collected from humans.

At present, nucleotide information for *Amphimerus* spp. DNA is very scarce, and only a few DNA partial sequences, corresponding to five isolates from hosts (including human, dog, cat, and two softshell turtles), are available in the Genbank database for potential LAMP primers design. The 459 bp sequence of the ribosomal DNA ITS2 region of *Amphimerus* sp. HS-2011 isolated from human hosts [4] was selected as a target of amplification. This sequence matches those later reported for isolates from a dog (dog-2012) and cat (cat-2012) residing in the same studied endemic indigenous Chachi communities for human amphimeriasis [4]. Therefore, the selection of the target region was appropriate because it seems to contain an identical sequence for all geographical isolates of *Amphimerus* spp. circulating in the same area, and the

assay could be suitable for easily diagnosing both infected animals and humans in endemic areas of amphimeriasis with limited resources.

First, we established the proper operation, sensitivity and specificity of both conventional PCR (using the outer primers) and the LAMP assay (using four specific primers: LAMPhimerus) in the amplification of the *Amphimerus* sp. DNA target sequence. Both assays were shown to be highly specific for *Amphimerus* sp. because no cross-reactivity could be observed when DNA from other parasites, including those closely related such as *C. sinensis* and *O. viverrini*, were used as a template in the reactions. Identical sensitivities (1 pg of parasite genomic DNA) were obtained for both PCR and LAMPhimerus although the LAMP technique is usually 10–100 fold more sensitive than PCR [38]. However, the sensitivity obtained was the same as that previously reported for *O. viverrini* detection targeting the ITS1 region in rDNA (ITS1-LAMP) [27, 29] or the mitochondrial *nad1* sequence (mito-OvLAMP) [28]. A higher sensitivity (10^{-5} pg) has been reported for detecting *C. sinensis* targeting the cathepsin B3 gene [12]. Perhaps, in this study, a greater sensitivity could have been achieved for our LAMPhimerus assay if other DNA target sequences for designing LAMP primers had been available to analyze in databases.

When PCR was specifically tested with the 44 field-collected stool samples, only 3 very faint PCR-positive results were obtained. Varying sensitivity of PCR detection for *O. viverrinii* [27] and *C. sinensis* [14] has already been noted when analyzing human stool samples because Taq DNA polymerase inhibitors are frequent in stool specimens. Substances typically present in human feces and dietary components can also limit DNA extraction success [39]. Therefore, improvement of DNA preparation before extraction from stool samples could be a key factor for obtaining better PCR results in *Amphimerus* sp. DNA detection, as has been previously described for other similar parasites, such as *O. viverrini* [40, 27]. In our study, the PCR assay is not emphasized because of its very low performance and inconvenience of application in poorly equipped and often short-staffed laboratories in endemic areas.

Better results were obtained when LAMPhimerus method was applied to test human stool samples. A better performance of LAMP assays over conventional PCR methods when analyzing stool samples has been widely reported in the literature because LAMP is more tolerant to sample-derived inhibitors than PCR for diagnostic applications [41, 42]. Therefore, using the initial established reaction time of 60 min, we obtained 14/44 (31.81%) positive results, including 9/25 (36%) that tested positive by microscopy. It has been already suggested that a longer incubation reaction time in the LAMP assay improves the sensitivity and that LAMP negative samples should be incubated longer to reduce false negatives [43]. According to this, a subsequent increase to 120 min of the standard incubation time protocol for the LAMPhimerus assay allowed us to increase the number of positives results up to 23/44 (52.27%), including 18/25 (72%) microscopy-confirmed *Amphimerus* sp. infections. It should be noted that 5 stool samples with no parasite eggs (nos. 36, 45, 47, 68 and 99) were positive on LAMPhimerus testing regardless of the reaction time used for amplification. These samples could be truly *Amphimerus* sp. infections that have been microscopically undetected because of the classically low sensitivity of the parasitological diagnosis [35]. Moreover, up to 10 samples without egg counts were also LAMPhimerus positive. This result confirms a greater sensitivity of the LAMPhimerus assay over microscopic examination.

By contrast, 7 truly microscopy *Amphimerus*-positive samples (nos. 34, 42, 46, 54, 62, 70 and 81) were never amplified. For these samples, values of FEC using the Kato-Katz thick smear method were minimal (between zero and 1–2 eggs), resulting in very low EPG levels. The absence of amplification in these samples was likely not due to the ineffectiveness of LAMPhimerus method because we obtained positive results in other microscopy-positive samples with low EPG levels too. A possibility for the lack of amplification could have been the small

quantity (250–300 mg) of the field-collected stool samples finally used for DNA extraction in the laboratory for the LAMP assay. Because eggs of parasites are not equally distributed among the stool specimens [44], it is possible that eggs could have been easily missed in working samples, compromising the *Amphimerus* sp. DNA obtained and thus subsequent amplification. It is also important to note that we established the minimum amount of *Amphimerus* sp. genomic DNA detectable by LAMP is 0.001 ng (1 pg). It has been reported that a single egg of a closely related trematode *O. viverrini* yields 3.72 ng of genomic DNA [45]. Then, theoretically, our LAMP assay would detect *Amphimerus* sp. DNA corresponding to less than one single egg in a stool sample. Another possibility could have been a mistake in the morphological identification of parasite eggs when performing the stool microscopic examination. This observation would further confirm the specificity of LAMPhimerus method in the amplification of *Amphimerus* sp. DNA alone.

However, as noted elsewhere, the need for a decision in case management dictates unequivocal result interpretation [22] and some of the drawbacks of LAMP assays, such as potential DNA contamination and carry-over of amplified products when opening the tubes to use the dye, should be considered because they may compromise the test results.

In summary, we have developed, for the first time, a LAMP assay (namely, LAMPhimerus) for the sensitive and specific detection of *Amphimerus* sp. DNA in human stool samples. After further research for validation, the method could be readily adapted for effective field diagnosis and disease surveillance in amphimeriasis-endemic areas. Future work will be aimed at large-scale studies to further assess the applicability of this novel diagnostic tool.

Supporting information

S1 Fig. Examination of human stool samples by PCR F3-B3. Analysis of human stool samples included in the study by PCR using outer primers F3 and B3 to detect *Amphimerus* sp. DNA. In all panels: lane Amp, DNA of *Amphimerus* sp. (10 ng); lane M, molecular weight marker (100 bp Plus Blue DNA Ladder); lane N, negative control (ultrapure water, no DNA template); and numbers 5–104, stool samples analyzed.
(TIF)

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Validation: WCT PFS MCH CFC AMA.

Visualization: WCT PFS MCH AMA.

Writing – original draft: WCT PFS MCH AMA.

Writing – review & editing: WCT PFS MCH HS AMA.

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3.5 ARTÍCULO 5: Diagnosis of amphimeriasis by LAMPhimerus assay in human stool samples long term storage onto filter paper.

William Cevallos, Pedro Fernández-Soto, Manuel Calvopiña, María Buendía-Sánchez, Julio López Abán, Belén Vicente, Antonio Muro.

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RESUMEN

La anfimeriosis es una enfermedad zoonótica transmitida por el consumo de peces crudos e infectados con el trematodo hepático *Amphimerus* spp. Es una enfermedad emergente recientemente reportada en un grupo Amerindio, los Chachis, que viven en Ecuador. El único método existente para diagnosticar la amphimeriosis era hasta la fecha la detección microscópica de huevos del parásito en muestras de heces de pacientes con muy baja sensibilidad. Nuestro grupo desarrolló una técnica ELISA para la detección de IgG anti-*Amphimerus* en suero humano y un método molecular basado en la tecnología LAMP (LAMPhimerus) para la detección específica y sensible del ADN del parásito. El método LAMPhimerus demostró ser mucho más sensible que los métodos parasitológicos clásicos para el diagnóstico de amphimeriosis utilizando muestras de heces humanas para el análisis. El objetivo de este trabajo es demostrar la viabilidad de utilizar muestras de heces secas conservadas en papel de filtro que sirvan como fuente de ADN en combinación con la eficacia de nuestro ensayo LAMPhimerus previamente diseñado para la detección de *Amphimerus* spp. en muestras clínicas de heces. Un total de 102 muestras de heces no tratadas, sin diluir, tomadas de la población Chachi, se extendieron como una fina capa sobre papel de filtro común para transportarlas fácilmente a nuestro laboratorio y almacenarlas a temperatura ambiente durante un año hasta la extracción del ADN. Cuando se aplicó el método LAMPhimerus para la detección de ADN de *Amphimerus* spp., se detectó un mayor número de resultados positivos (61/102; 59,8%) en comparación con los métodos parasitológicos (38/102, 37,25%), incluyendo 28/61 (45,9%) infecciones de *Amphimerus* spp. confirmadas por microscopía. La sensibilidad y especificidad del diagnóstico para nuestro ensayo LAMPhimerus, fueron 79.17% y 65.98%, respectivamente. Demostramos, por primera vez, que el papel de filtro común es útil para la recolección fácil y el almacenamiento a largo plazo de muestras de heces humanas para la posterior extracción de ADN y el análisis molecular de huevos de trematodos parásitos de humanos. Este método simple, económico y, de fácil manejo, combinado con el ensayo LAMPhimerus específico y sensible, tiene el potencial de ser utilizado como una prueba de análisis molecular efectiva a gran escala para áreas endémicas de amphimeriosis.

PLOS ONE

RESEARCH ARTICLE

Diagnosis of amphimeriasis by LAMPhimerus assay in human stool samples long-term storage onto filter paper

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Abstract

Amphimeriasis, a fish-borne zoonotic disease caused by the liver fluke *Amphimerus* spp., has recently been reported as an emerging disease affecting an indigenous Ameridian group, the Chachi, living in Ecuador. The only method for diagnosing amphimeriasis was the microscopic detection of eggs from the parasite in patients' stool samples with very low sensitivity. Our group developed an ELISA technique for detection of anti-*Amphimerus* IgG in human sera and a molecular method based on LAMP technology (named LAMPhimerus) for specific and sensitive parasite DNA detection. The LAMPhimerus method showed to be much more sensitive than classical parasitological methods for amphimeriasis diagnosis using human stool samples for analysis. The objective of this work is to demonstrate the feasibility of using dried stool samples on filter paper as source of DNA in combination with the effectiveness of our previously designed LAMPhimerus assay for successfully *Amphimerus* sp. detection in clinical stool samples. A total of 102 untreated and undiluted stool samples collected from Chachi population were spread as thin layer onto common filter paper for easily transportation to our laboratory and stored at room temperature for one year until DNA extraction. When LAMPhimerus method was applied for *Amphimerus* sp. DNA detection, a higher number of positive results was detected (61/102; 59.80%) in comparison to parasitological methods (38/102; 37.25%), including 28/61 (45.90%) microscopy-confirmed *Amphimerus* sp. infections. The diagnostic parameters for the sensitivity and specificity werecalculated for our LAMPhimerus assay, which were 79.17% and 65.98%, respectively. We demonstrate, for the first time, that common filter paper is useful for easy collection and long-term storage of human stool samples for later DNA extraction and molecular analysis of human-parasitic trematode eggs. This simple, economic and easily handling method combined with the specific and sensible LAMPhimerus assay has the potential to beused as an effective molecular large-scale screening test for amphimeriasis-endemic areas.

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Introduction

Amphimeriasis, a fish-borne zoonotic disease caused by the liver fluke *Amphimerus* spp. (within the family Opisthorchiidae), was recently reported as an endemic disease in the tropical Pacific side of Ecuador. Data showing high prevalence of infection among an indigenous group, the Chachis, and also domestic cats and dogs residing in the same communities have been noted and, actually, human amphimeriasis has been reported as a new emerging food-borne zoonotic disease. Parasites of the genus *Amphimerus* infect humans after ingestion of raw or undercooked freshwater fish containing viable metacercariae. Human disease is mostly asymptomatic, occasionally causing non-specific, generalised symptoms. However, histopathological studies in cats and a double-crested cormorant infected with *Amphimerus* spp. showed the presence of liver cirrhosis and pancreatitis [1,2]. Similarly, as occur in other human infections by parasites of the family Opisthorchiidae, affected individuals with *Amphimerus* spp. can suffer from suppurative cholangitis, cholelithiasis and cholangiocarcinoma [3–5]. Since the Chachi community habitually consumes smoked freshwater fish, an estimated 13% of the inhabitants living along the Rio Cayapas in the Province of Esmeraldas are a risk of acquiring amphimeriasis [6].

Until very recently, the only method for diagnosing the disease was the microscopic detection of eggs from the parasite in patients' stool samples, but it lacks in sensitivity [6]. To overcome this limitation, our investigation group developed, for the first time, an ELISA technique for detection of anti-*Amphimerus* IgG in human sera [7] and, afterwards, the first molecular method based on LAMP technology (named LAMPhimerus) for specific and sensitive parasite DNA detection. The LAMPhimerus method showed to be much more sensitive than the classical parasitological methods for amphimeriasis diagnosis using human stool samples for analysis [8]. In that study, a number of human stool samples from Chachi communities were preserved in 80% ethanol solution for later DNA extraction to test by LAMP assay. It is known that collection of fresh stool samples for diagnostic purposes can be quite difficult in some population groups. Besides, the handling, management and storage of a large number of patients' stool samples can be very laborious in large-scale field trials in poor settings with minimal infrastructures. This fact is especially true for many tropical diseases since they are frequently in populations remote from sophisticated diagnostic facilities. Dried samples spots or smears collected onto filter paper provide a potentially useful and economic means of overcoming these drawbacks. The use of dried specimens -especially blood and sera samples- for the diagnosis and surveillance of infectious diseases has been recently reviewed [9]. In general, dried specimens perform with sensitivities and specificities very similar to gold standard sample types when using for DNA extraction and subsequent analysis by PCR-based molecular methods. However, a standardization methodology is still needed. For collection, preservation and easy handling of stool samples onto filter paper there are very few cases, and only including protozoa studies [10–13].

It should be very interesting to join the advantages of using filter paper for easy collection and preservation of human stool samples and the easy LAMP technology [14]. Considering a number of salient advantages of LAMP over most PCR-based molecular methods [15, 16], LAMP technology shows a potential use in clinical diagnosis and surveillance of infectious diseases, particularly under field conditions in developing countries for most tropical diseases [17, 18].

As mentioned above, we have recently developed a sensible and specific LAMP assay for the successful detection of *Amphimerus* sp. DNA in human stool samples from a Chachi community. Now, the objective of this work is to demonstrate the feasibility of using dried stool samples on filter paper as source of DNA in combination with the effectiveness of our

previously designed LAMPhimerus assay for successfully *Amphimerus* sp. detection in clinical stool samples.

Materials and methods

Ethics statement

The study protocol was approved by the Ethics Committee of Universidad Central del Ecuador (License number: LEC IORG 0001932, FWA 2482, IRB 2483. COBI-AMPHI-0064-11) and the Ethics Committee of the University of Salamanca (protocol approval number 48531). Participants were given detailed explanations about the aims, procedures and possible benefits of the study. Written informed consent was obtained from all subjects prior to the collection of biological samples for parasitological and molecular evaluation. Parents or guardians of children who participated in the study provided written informed consent on the child's behalf. All samples were coded and treated anonymously. Procedures were performed in accordance with the ethical standards laid down in the Declaration of Helsinki as revised in 2013.

Study area and population

The study was conducted during February 2016 in two indigenous Chachi villages (El Progreso and Estero Vicente) in the Canton Eloy Alfaro alongside the Cayapas River in the Esmeraldas province, located in the northwest coastal rainforest of Ecuador, 320 km from the capital Quito. The indigenous Chachi -living together with Afro-ecuadorian and mestizo populations- is the predominant autochthonous group in this area, representing 13% of the inhabitants in this region. In these Chachi communities high prevalence of human (15.5% to 34.1%) and local cats and dogs (71.4% and 38.7%, respectively) with *Amphimerus* spp. have been previously reported [6, 19]. They live in remote villages where the only way to reach them is by boat along the river. Sanitation facilities are lacking. The members are hunters who typically eat undercooked freshwater fish (mainly smoked fish) caught in the neighboring rivers and food sharing is usually common [6, 19]. The main economic activities are agriculture, fishing and exploitation of forest resources. The province of Esmeraldas, forms part of the tropical rainforest known as "Choco Biogeográfico del Pacífico" which covers a section of the coast of Ecuador, Colombia and Panamá. This area has been labeled as a biological hotspot, an area with an extraordinary concentration of animal species. More details on the region can be accessed elsewhere [20].

Human stool sampling and parasitological tests

A total of 102 participants living in two indigenous Chachi communities were enrolled in the study, including 56 females (54.90%) and 46 males (45.09%) with a median age of 20.39 (range 1–65 years). Each participant was given a copro-parasitological flask for stool collection. Samples were collected within a few hours of stool passing. A single stool sample was individually obtained from each participant. After collection, samples were transported to the laboratory of parasitology (Centro de Biomedicina, Universidad Central del Ecuador, Quito, Ecuador) for parasitological screening under light microscopy by simple sedimentation technique (SST), formalin-ether concentration technique (FECT) and Kato-Katz technique (KKT). All samples were examined by two qualified laboratory technicians according to the basic laboratory procedures in Medical Parasitology, recommended by the World Health Organization (WHO) [21].

In addition, a portion of each sample was spread with a swab onto a filter paper (10 X 2 cm, approximately), air-dried, numbered, folded in a half, and individually wrapped in foil. In that

way, samples were stored at room temperature until shipped to the Research Centre for Tropical Diseases at the University of Salamanca, Spain, for further DNA extraction (during February 2017) and molecular analysis as described below.

DNA extraction for molecular analyses

DNA from parasites. *Amphimerus* sp. genomic DNA was extracted from frozen adult worms that were previously obtained from the livers of naturally infected cats and dogs of Chachi communities, as described elsewhere [19], using a G-spin Total DNA Extraction Kit (Intron Biotechnology) according to the manufacturers' instructions. DNA was measured using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies) and then diluted with ultrapure distilled water to final concentration of 0.5 ng/μL to use as positive control in all LAMP reactions.

DNA from human stool samples smeared on filter papers. DNA from human stool samples smeared on filter papers was extracted 12 months after collection and preparation. Steps followed for DNA extraction are shown in Fig 1. DNA extraction procedure was performed in batches of 10 samples each for easy handling and also to prevent potential cross-contamination. DNA extraction was performed using the i-genomic Stool DNA Extraction Mini Kit (Intron Biotechnology) according to the manufacturers' instructions with some additional procedures as follows. The smeared portion of filter papers were cut with scissors into thin strips. Scissors were always sterilized before cutting the next sample to prevent contamination. Thin strips of each sample were first placed into a 1.5 mL tube immersed in a lysis mixture -TE (400 μL; pH 8.0), lysis buffer (200 μL Buffer SL) and proteinase K (20 μL)-, vortexed vigorously, and subsequently incubated for 30 min at 65˚C in a thermoblock. During incubation, to help dissolve feces until complete lysis the tubes were vortexed or inverted at about

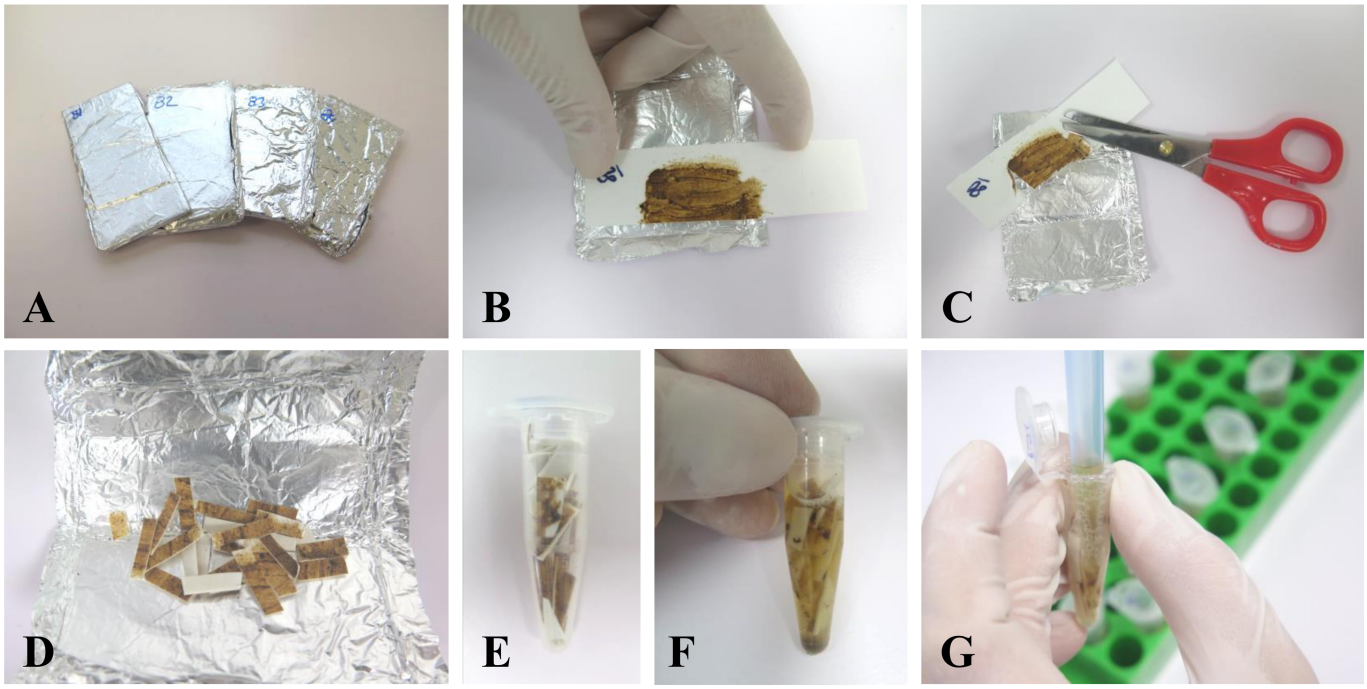

Fig 1. Human stool samples processing for DNA extraction. A. Batches organization. B. Smeared stool sample on filter paper. C, D. Filter paper is cut with scissors into thin strips. E, F Thin strips of each sample are placed into a 1.5 mL tube immersed in a lysis mixture an incubated for 30 min at 65˚C in a thermoblock G. Mixture is transfer for begining DNA extraction with the commercial kit i-genomic Stool DNA Extraction Mini Kit (iNtRON Biotechnology).

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5–10 min intervals. After incubation, a volume of 500 μL approximately of the mixture was transferred into a IR Spin Column for proper binding, washing, and elution steps. A final eluate of 100 μL of genomic DNA (gDNA) was obtained from each sample and divided into two aliquots of 50 μL each. After measuring the concentration using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies), DNA samples were stored at -20˚C until use in molecular assays.

LAMPhimerus assay

All the human stool samples were tested using the reaction mixture and specific primer set for LAMP assay (LAMPhimerus) previously established by our group [8]. The LAMPhimerus method amplifies a sequence of the *Amphimerus* sp. internal transcribed spacer 2 region (GenBank acc. no. AB678442.1). Briefly, the reaction was carried out with a total of 25 μL reaction mixture containing 40 pmol of each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 1.4 mM of each dNTP (Intron), 1x Isothermal Amplification Buffer -20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20- (New England Biolabs, UK), 1 M betaine (Sigma, USA), supplementary 6 mM of $MgSO_4$ (New England Biolabs, UK) and 8 U of *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs, UK) with 2 μL of template DNA. Reaction tubes were placed in an economic heating block (K Dry-Bath) at a constant temperature of 63˚C for 90–120 min and then heated at 80˚C for 5 min to stop the reaction. In all LAMPhimerus trials positive controls (*Amphimerus* sp. gDNA) and a negative controls (water instead DNA) were included.

The LAMP amplification results could be visually inspected by the naked eye by colour change after adding 2 μL of 1:10 diluted 10,000X concentration SYBR® Green I (Invitrogen) to the reaction tubes. To avoid as much as possible, the potential risk of cross-contamination with amplified products, all tubes were briefly centrifuged and carefully opened before adding the fluorescent dye. Green fluorescence was clearly observed in successful LAMP reaction, whereas it remained original orange in the negative reaction. The LAMP products (3–5 μL) were also monitored using 1.5–2% agarose gel electrophoresis, visualized under UV light and then photographed using an ultraviolet image system (Gel documentation system, UVItec, UK).

Statistical analysis

To estimate the accuracy of the LAMP assay method as a diagnostic test, the percentages of the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using the MedCalc statistical program version 16.8.4 (MedCalc Software, Ostende, Belgium) according to the software instruction manual (http://www.medcalc.org).

Results

Parasitological tests

Of the total of 102 stool samples examined microscopically for the presence of *Amphimerus* eggs, 38 (37.25%) resulted positive at least by one of the parasitological techniques applied, including 27 (26.47%) positive by the simple sedimentation technique (SST), 19 (18.62%) positive by the formalin-ether concentration technique (FECT), and 27 (26.47%) positive by the Kato-Katz technique (KKT). Up to 15 (15/102; 14.70%) stool samples resulted simultaneously positive for the three parasitological tests; only 3 (3/102; 2.94%) stool samples resulted simultaneously positive for two parasitological tests, including 1 for SST and KKT and 2 for SST and FECT.

LAMPhimerus analysis

Amplification assays were performed in batches of 10–11 samples each for easy handling and to prevent potential cross-contamination. All the samples were analyzed in duplicate with identical result. We obtained LAMP positive results in 61/102 (59.80%) samples, including 33/61 (54.09%) samples that were negative in all parasitological tests applied and 28/61 (45.90%) samples that were positive at least by one of the parasitological technique applied. Of the 15 samples (nos. 6, 27, 28, 30, 31 32, 33, 42, 54, 60, 79, 84, 85, 93, 97) that were simultaneously positive on three parasitological tests (FECT, SST and KKT), up to 13 (13/15; 86.66%) (nos. 27, 28, 30, 31 32, 33, 42, 54, 60, 79, 84, 85, 93) were also positive by LAMPhimerus assay; only 2 samples (2/15; 13.33%) (nos. 6 and 97) were negative on the LAMPhimerus assay. In all LAMP positive amplifications, green fluorescence was clearly visualized under natural light conditions and also by electrophoresis in agarose gels (Fig 2). Positive controls always worked well and negative controls were never amplified.

In Fig 3 a total comparison of the results obtained by LAMPhimerus assay and parasitological techniques applied for detecting *Amphimerus* sp. in human stool samples is showed.

Considering the microscopy findings by parasitological techniques as the reference standard, the following diagnostic parameters for the sensitivity and specificity were calculated for our LAMPhimerus assay in this study: 79.17% sensitivity (95% CI: 65.0% -89.53%); 65.98% specificity (95% CI: 55.66% -75.30%); 53.52% positive predicted value (95% CI: 45.72% -61.16%) and 86.49% negative predicted value (95% CI: 78.36% -91.88%).

Discussion

The indigenous Chachi communities, who live in remote villages along the Río Cayapas in the north-western coastal rainforest of Ecuador, have been shown to have a high prevalence of infection (15.5%-34.1%) with *Amphimerus* sp. [6]. Infection in domestic cats and dogs residing in this endemic area has also been reported as high (71.4% and 38.7%, respectively) and these animals have been proposed to serve as definite hosts and reservoirs for the parasite [19]. The prevalence data obtained in these studies were assessed according to eggs findings in both human and animal stool samples by classical parasitological methods.

Recently, in a pilot study using 44 human stool samples preserved in 80% ethanol solution from that area, a novel LAMP assay (LAMPhimerus) showed to be more sensitive than parasitological techniques for diagnosing human amphimeriasis [8]. Therefore, LAMPhimerus was proposed as a new molecular tool that could be readily adaptable for effective field diagnosis in amphimeriasis-endemic areas. However, the handling, management and storage of a large numbers of patients' fresh or frozen stool samples for diagnosing amphimeriasis in remote areas with poor infrastructure can be very difficult in large-scale field trials. Filter paper potentially provides a useful medium to overcome a number of difficulties of fresh sample collection, preservation and transportation. This method has been widely used as a specimen substrate when performing diagnostic or epidemiological surveys, especially in remote areas in resource-poor settings [9]. However, most studies have used filter papers for blood and sera collection and studies applying this method in human faecal samples for subsequent molecular detection of parasites are still very limited; a few reported examples are *Enterocytozoon bieneusi* [10, 11], *Giardia duodenalis* [12] and *Blastocystis* spp. [13]. Thus, the aim of this work is to demonstrate the feasibility of using filter paper for collection and preservation of human stool samples as source of DNA in combination with the effectiveness of our previously designed LAMPhimerus assay for successfully *Amphimerus* sp. detection in clinical stool samples.

There are several kinds and brands of filter paper available consisting of 100% cellulose and varying in thickness and pore size that have been used in different studies for PCR-based

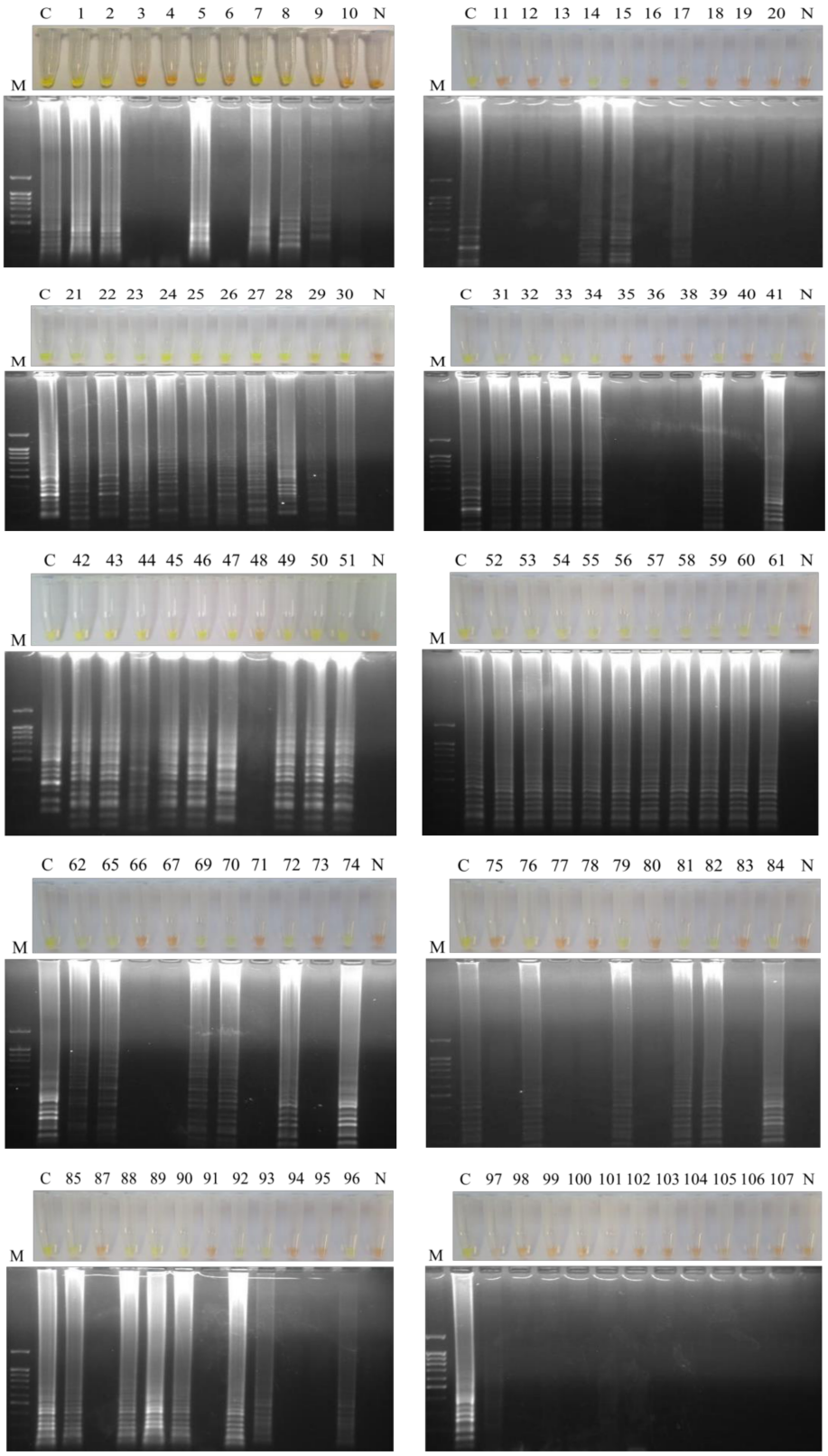

Fig 2. LAMPhimerus analysis of human stool samples in this study. Lanes M, molecular weight marker (100 bp Plus Blue DNA Ladder); lanes C, *Amphimerus* sp. genomic DNA (1 ng); lanes N, negative controls (ultrapure water and no DNA template); numbers 1–107, analyzed human stool samples.

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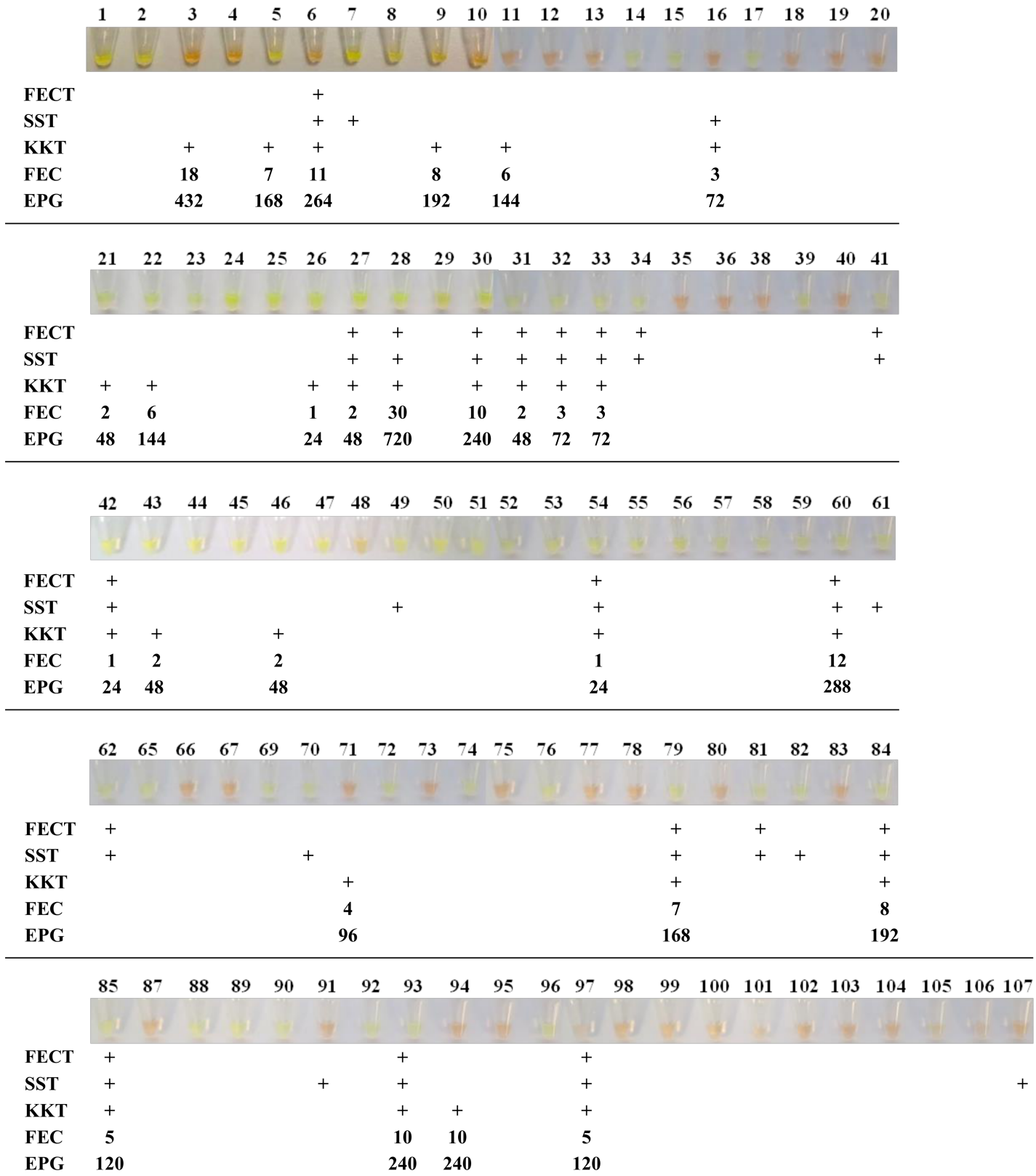

Fig 3. Comparison of the results obtained by the LAMPhimerus assay and classical parasitological techniques applied in this study. FECT, formalin-ether concentration technique; SST, simple sedimentation technique; KKT, Kato-Katz technique; FEC, fecal egg count; EPG, eggs per gram of feces; +, positive for egg detection. Values indicated for FEC and EPG correspond to the numbers of detected eggs. Numbers 1–107 correspond to the analyzed human stool samples.

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detection of DNA from humans, plants, animals, viruses, bacteria and parasites [9, 13]. In some cases, filter papers are impregnated with a proprietary mix of chemicals which provide protection of DNA of samples thus avoiding degradation and subsequent successful extraction. In addition, filter paper technology, such as FTA (Flinders Technology Associates)-treated matrix cards, may inactivate highly pathogenic organisms for safety transporting and long-term storage [13, 22]. However, some disadvantages of FTA paper are the use of a restricted diluted faecal sample volume of 15 μL for detection of protozoa and the whole procedure to get DNA template ready for PCR amplification takes approximately 3 hours [11].

In our preservation method, we used an economic common filter paper (100% cellulose with smooth surface and normal hardness) which is used for routine laboratory procedures such as basic filtration. Untreated and undiluted stool samples were spread as thin layer onto the filter papers for easily transportation to our laboratory and stored at room temperature for one year until DNA extraction. In our case, the whole procedure to get DNA template ready for LAMPhimerus assay, including the cutting of the strips, pre-incubation with the lysis mixture and DNA extraction with the commercial kit, can be performed in just 45 min. In addition, when measuring the DNA concentration of samples, the procedure yielded enough quantity of quality DNA for molecular detection by LAMPhimerus assay. According to this, long-term storage of dried stool samples onto common filter paper at room temperature worked very well for subsequent DNA extraction.

Thus, when LAMPhimerus method was applied to test human stool samples for *Amphimerus* sp. DNA detection, a higher number of positive results was detected (61/102; 59.80%) in comparison to parasitological methods (38/102; 37.25%), including 28/61 (45.90%) microscopy-confirmed *Amphimerus* sp. infection. It is important to note that up to 33/61 (54.09%) samples that were negative in all parasitological tests applied were LAMPhimerus-positive. These samples could be truly *Amphimerus* sp. infections undetected because of the known classically low sensitivity of the microscopy diagnosis in trematode infections [23]. This data reinforces the previous greater sensitivity of the LAMPhimerus assay over microscopic examination when testing human stool samples preserved in 80% ethanol solution [8]. On the other hand, only 8 truly parasitological *Amphimerus*-positive samples (nos. 3, 6, 11, 16, 71, 94, 97 and 107) were never amplified by LAMPhimerus assay. We think that the inoperative amplification in these samples was not due to the ineffectiveness of LAMPhimerus method because we obtained positive results in other microscopy-positive samples with lower EPG levels. Besides, the minimum amount of *Amphimerus* sp. genomic DNA detectable by LAMPhimerus assay (1 pg) has been reported to correspond to less than one single egg of the parasite in a stool sample [8]. An explanation for the inoperative amplification could be that the amount of the sample onto the filter paper was not enough to obtain *Amphimerus* sp. DNA for analysis. Perhaps, an inaccuracy in microscopy identification of parasite eggs occurred since morphological similarity of the *Amphimerus* spp. eggs to those of closely related species belonging to Opisthorchiidae family and to minute intestinal flukes makes diagnosis very difficult. Sometimes, it is necessary to use scanning electron microscopy to accurately observe the differences between the coatings on the different species [6]. This observation would further reinforce the specificity of LAMPhimerus method in the solely amplification of *Amphimerus* sp. DNA.

Conclusions

In conclusion, to the best of our knowledge, we demonstrate for the first time that common filter paper is useful for long-term storage of human stool samples for later quality DNA extraction of human-parasitic trematode eggs. Additionally, this simple, economic and easily

handling method combined with the specific and sensible LAMPhimerus assay has the potential to be used as an effective molecular large-scale screening test for amphimeriasis-endemic areas. The system 'air-dried stool sample on filter paper'-LAMP assay could also be very interesting and useful for molecular diagnosis of other human infectious parasitic diseases in remote areas with poor settings.

Supporting information

S1 Checklist. STARD checklist.
(DOCX)

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4. CONCLUSIONES

CONCLUSIONES

1. Se describe por primera vez la amphimeriosis como infección humana causada por el trematodo hepático *Amphimerus* spp. en humanos residentes en la selva tropical húmeda del Ecuador. Anteriormente solo se conocía la infección en varias especies de animales domésticos y silvestres en varios países de las Américas y Asia.

2. La prevalencia de *Amphimerus* spp. en personas residentes en las comunidades indígenas Chachis del Noroeste de Ecuador estudiadas es elevada, llegando hasta el 34% de infección.

3. Los animales domésticos -gatos y perros- que habitan en las comunidades estudiadas también presentan altos niveles de infección por *Amphimerus* spp. (71,4% y 38,7%, respectivamente) demostrando que actúan como hospedadores definitivos y reservorios del parásito. De esta manera, se evidencia el carácter zoonótico de esta tramatodosis.

4. La técnica ELISA desarrollada por primera vez para la detección de anticuerpos IgG anti-*Amphimerus* spp., presenta alta sensibilidad (85%). Se requieren futuros estudios para mejorar su rendimiento utilizando moléculas más específicas.

5. Se ha desarrollado y evaluado por primera vez un método molecular basado en la tecnología LAMP (LAMPhimerus) útil para el diagnóstico de la amphimeriosis en muestras de heces humanas, presentando una sensibilidad del 76,7% y una especificidad del 80,8%.

6. El uso de papel de filtro para la recolección y conservación de muestras de heces humanas durante largos períodos de tiempo a temperatura ambiente ha resultado ser un método efectivo para la posterior aplicación del método LAMP.

7. El análisis de muestras de heces almacenadas sobre papel de filtro mediante LAMPhimerus es de gran utilidad para el diagnóstico de la amphimeriosis en condiciones de campo y sería de gran utilidad en la vigilancia epidemiológica de esta parasitosis.

5. OTROS ARTÍCULOS DE INVESTIGACIÓN

5.1 Sensibilidad de la técnica de Kato-Katz para la detección de huevos de *Amphimerus* en muestras de heces, y prevalencia de infección en Amerindios Chachis.

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RESUMEN

El presente estudio compara la sensibilidad de cuatro métodos coproparasitarios diferentes para detectar huevos del trematodo hepático *Amphimerus* spp. en heces y evidencia la prevalencia de infección en residentes indígenas Chachi residentes en un bosque tropical lluvioso de la costa Noroeste de Ecuador. Se examinaron un total de 105 muestras aplicando las técnicas de Kato-Katz (KK), sedimentación simple en tubo (SSTT), concentración de formalina-éter (FEC) y microscopía de frotis directa (DM). La sensibilidad de cada método fue del 25.7% (IC 95%: 17.5-34%), 21% (IC 95%: 13.1-28.7), 18% (IC 95%: 10.7-25.3) y 1 % (95% CI: 0.9-2.9), respectivamente. Combinando los cuatro métodos, 38 muestras fueron positivas con una prevalencia de infección de 36,1%. Nuestros resultados indican que el KK solo obtuvo el mejor resultado y detectó 27 (71%) de las 38 muestras positivas. Combinando KK y SSTT se diagnosticó amphimeriosis en 35 muestras (92.1%) y en 31 muestras (81.6%) mediante KK y FEC. La técnica de SSTT sola, detectó 22 muestras (57.9%) y sería recomendado realizar esta técnica en estudios de campo debido a su simplicidad. La menor sensibilidad mostrada por el método de DM, plantea una seria preocupación. Realizar dos técnicas en una sola muestra mejora la detección de la infección por *Amphimerus* spp.

SENSITIVITY OF THE KATO-KATZ TECHNIQUE IN COMPARISION TO THREE OTHER DIAGNOSTIC METHODS FOR THE DETECTION OF *AMPHIMERUS* EGGS AND THE PREVALENCE OF INFECTION IN CHACHI AMERINDIANS IN ECUADOR

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Abstract

The present study compares the sensitivity of four different coproparasitological methods for detecting eggs of *Amphimerus* spp. liver fluke in stools and evidenced the prevalence of infection in indigenous Chachi residents in a tropical rain forest in the northwest coast of Ecuador. A total of 105 samples were examined applying the Kato-Katz technique (KK), the spontaneous sedimentation technique in tube (SSTT), the formalin-ether concentration technique (FEC), and direct smear microscopy (DM). The sensitivity of each method was 25.7% (95% confidence interval (CI): 17.5–34%), 21% (95% CI: 13.1–28.7), 18% (95% CI: 10.7–25.3), and 1% (95% CI: 0.9–2.9), respectively. Combining the four methods, 38 samples were positive with a prevalence of infection of 36.1%. Our results indicated that KK alone performed the best, detecting 27 (71%) of the 38 positive samples. Combining KK and SSTT, amphimeriasis were diagnosed in 35 samples (92.1%), and in 31 samples (81.6%) by KK and FEC methods. SSTT alone detected 22 samples (57.9%) and would be recommended for field studies because of its simplicity. The lowest sensitivity showed by the DM method raises a concern. Performing two techniques on a single sample enhanced the detection of *Amphimerus* infection.

INTRODUCTION

A high prevalence of human infection by the *Amphimerus* spp. liver fluke, was recently reported in the indigenous population of Chachi located in the province of Esmeraldas, in the northwest region of Ecuador. The prevalence, which varied from 15.5% to 34.1%, was closely associated with the distance inland from the coast (Calvopina et al. 2011). More recently, a higher prevalence of infection in cats and dogs living in the same communities where humans are infected, was evidenced (Calvopina et al. 2015). *Amphimerus* is a trematode belonging to the Opisthorchiidae family, along with the *Clonorchis sinensis* and *Opisthorchis* spp., which can also affect the bile ducts but are only endemic in Asian countries. Liver fluke infection is one of the more important food-borne diseases worldwide and is considered by the World Health Organization as a neglected tropical disease (WHO, 2010). *C. sinensis* and *Opisthorchis* spp. are classified by the International Agency for Research on Cancer (IARC) as carcinogenic group 2A

and class 1, respectively considering the high association of their presence and the development of cholangiocarcinoma (Keiser and Utzinger, 2009).

The genus *Amphimerus* Barker, 1911 (Trematoda: Opisthorchiidae) had been described mainly in the American continent including Canada, USA, Costa Rica, Panamá, Colombia, Venezuela, Ecuador, Brazil and Perú (Calvopiña et al. 2011) as well as from South Korea, India and the Philippines (Eom et al. 1984, Yamaguti 1958). There are no known reports of its presence in other continents such as Africa, Oceania, and Europe. Adult worms are hermaphrodites and parasitize the bile ducts and gall bladder, eliminating their eggs via stools. Consequently, microscopy examination of stool samples searching for eggs is the most commonly used technique for diagnosing *Amphimerus* infection (Calvopina et al. 2011 & 2015). Other diagnostic immunological and molecular methods have been developed very recently, ELISA and LAMPhimerus, showing some sensitivities of 85% and 76.6%, and some specificities of 71% and 80.7%, respectively. (Cevallos et al. 2017a; Cevallos et al 2017b).

In the previous studies on human and domestic animals in Ecuador, diagnosis was made by microscopic observation of *Amphimerus* eggs in stools (Calvopina et al. 2011 & 2015). The only coproparasitological technique used was the formalin-ether concentration (FEC) because the direct smear microscopy (DM) showed low sensitivity (Calvopina et al. 2011). DM is the most commonly used method in the public and private laboratories for parasites examination of stools in Ecuador and neighboring countries.

For the detection of human helminth parasites, where their eggs are eliminated via stools, WHO currently recommends the use of the Kato-Katz (KK) method (WHO, 2002). Other commonly used methods include direct smear microscopy, formalin-ether concentration, formalin-ethyl acetate, McMaster, FLOTAC and mini-FLOTAC (WHO, 2002). Spontaneous sedimentation technique in tube (SSTT) has been described by Tello (1988) (This article doesn't appear in the Reference section) being a coproparasitological technique with high sensitivity to detect the majority of intestinal parasites, including eggs, larvae, cysts, and trophozoites and required less costly materials and equipment (Tello et al. 2012). Sedimentation techniques use solutions of lower specific gravity than the parasitic organisms, thus concentrating eggs and larvae in the sediment.

Sedimentation techniques are recommended for general diagnostic laboratories because they are easier to perform and less prone to technical errors (CDC, 2013). All of these techniques rely on visual examination of prepared stool samples under a light microscopy. With the exception of SSTT all of the others use a small amount of fecal material.

For *Amphimerus* liver fluke there is a lack of studies comparing detection techniques to the KK method. Hence, we conducted a study to determine the best method, or combination of methods, to detect the eggs of *Amphimerus* in stools under field conditions of individuals in a remote amphimeriasis endemic area and to determine the prevalence of *Amphimerus* infection in a Chachi community-based sample.

MATERIALS AND METHODS

Study design and area. This is a cross-sectional study performed in a remote village alongside the Cayapas river, located in the northwestern coastal region of Ecuador, in the province of Esmeraldas, canton Eloy Alfaro, latitude of 0.721283°, longitude of -78.906783°, at an elevation of 34 meters above sea level. The only means of transportation to reach this community is by boat and is located approximately 131 Km inland from the coast. The ecosystem, characteristics of this region, is described elsewhere in previous studies (Sierra 1999, Calvopina et al. 2011 & 2015; Eisenberg et al. 2006)

Study population. The study was first socialized and discussed with the local Chachilla in a general assembly, where they were informed of the objectives of the study. Family leaders were asked that all family members participate in the study by providing a stool sample taken in the next three days. People were provided with a plastic stool flask collector. A community health worker translated the information into the local language "cha´palaachi". Chachi indigenous group are Amerindians and represent around 13% of the 24,000 inhabitants in the region. Fishing and farming are their main activities.

Stool collection and parasitological examination. The study was conducted in August 2015. Within a few hours of stool collection, each stool specimen was processed as follow: 1) for the direct smear method (DM), a wet mount was prepared with approximately 20 mg of feces suspended in Lugol's staining solution and observed microscopically; 2) a single KK thick smear, 3) five grams of fecal material was suspended in 10 mL of warm saline solution, and 4) a smear was spotted in a FTA card filter paper, 3 x 2 cm in size and preserved at room temperature for future studies. All samples, except for formalin-ether sedimentation (FEC), were processed, observed under light microscopy at the magnification of 100x and 400x and results were recorded, while in the community. The remaining amount of fecal material was preserved in 70% ethanol and 10% formalin and transported to the parasitology laboratory at Centro de Biomedicina in Quito, Universidad Central del Ecuador, where it was used for FEC.

Kato Katz (KK) technique. A 3% methylene blue-glycerol solution was prepared in advance. Cellophane strips of the size of a slide were cut and immersed in the solution for 24 hours before their use. The KK technique was performed following the WHO protocol. A single thick smear slide was prepared using 41.7 mg punched plastic templates. *Amphimerus* egg count were recorded as total count and then converted to eggs per gram (EPG) of stool as appropriate.

The EPG was obtained by multiplying the number of eggs per slide by 24 (WHO, 2002).
Formalin-ether concentration (FEC). The procedure followed was a modified protocol described by Ash and Orihel (1991). Briefly, 3 grams of stool was weighed, homogenized, and diluted to 14 mL with 10% formalin in a 15 mL plastic tube. After manually shaking the tube to mix the fecal content, the mixture was filtered throughout a double surgical gauze into a second conical screwed 15 mL plastic tube. The supernatant was manually

decanted and the plastic tube with its content allowed to stand for 10 minutes. After the addition of 3 mL ethyl ether (Fisher Chemical, New Jersey, USA), the tube was filled up till 10 mL with 10% formalin, capped and vigorously agitated for 30 seconds by hand. The sample was centrifuged at room temperature at 2500 rpm for 5 minutes. This procedure assured the formation of three layers within the tube. The upper 2 layers were decanted and then 50 µl of 10% formalin was added to the sediment. Approximately 50 µl of sediment was placed on a slide and covered with a cover slip. A small drop of Lugol´s staining solution was placed between the slide and cover slip and then examined under the light microscope; first at 100x and confirming the observation with 400x magnification.

Spontaneous Sedimentation Technique in Tube (SSTT). The protocol used was that described by Tello (1988) with some modifications. Briefly, five grams of fresh feces were weighed and homogenized by strong manual agitation in a 50-mL plastic tube containing 25 mL of 0.9% saline (Na Cl) solution. It was then filtered through a double-layer surgical gauze and poured to another clean 50-mL plastic tube and filled up to 45 mL with warm (40^{o}C) saline and mixed again for around 30 seconds. If the feces consistency was hard, it was macerated by a wooden tongue depressor and left upright at room temperature for 2 hours. The supernatant was manually decanted and a sample of the sediment was removed with a plastic pipet. The sample was placed on a glass slide with Lugol´s staining solution, covered with a cover slip and observed under a light microscopy (100x and 400x).

Two laboratory technicians observed the samples for the 4 different methods and one expert (MC) verified and made an external control of the slides. An individual was considered positive for *Amphimerus* infection if one or more eggs were observed by one or more of the methods employed.

Statistical analyses

Since a "gold" standard test is not available for the detection of an *Amphimerus* infection, the operational characteristics (sensitivity and negative predictive values (NPV) were estimated using the combined results from the four methods employed as the "gold" standard (Joseph J. et al. 1995; Dendokuri et al. 2004; Goodman et al. 2007). Data were analyzed using SPSS version 16 and JavaStat software. Sensitivities, NPV, and kappa were determined for the various tests to evaluate their operational characteristics. *P* values <0.05 were considered statistically significant. The sensitivity of each method and combinations of methods were calculated based on comparison with those results obtained by all methods combined.

The sensitivity and negative predictive value (NPV) were assessed for each diagnostic technique in comparison to a composite reference standard, which was defined as being positive if any of the 4 tests were positive.

Ethics

Signed consent was obtained by the leaders and school teachers of the community and informed consent by each participant and from the guardians if children were enrolled. Individuals were free to refuse their participation and its delivery of stool samples. Ethical committee of the Universidad Central del Ecuador reviewed and approved this study (license number LEC IORG 0001932, FWA 2482, IRB 2483. COBI-AMPH-0064-11). In a second visit to the community, all participants positive for any kind of parasites, were treated with antiparasitic medication following the guidelines of Ecuadorian Ministry of Public Health.

RESULTS

The total population of the study village was 135 Chachi inhabitants. Of them, a total of 107 individual samples were collected; two were discarded due to inadequate amount of fecal matter. Of the participating individuals 59 were females and 46 males, with a mean age of 21.7 years, ranging from 1 to 65 years; 37% were of the 1-9 age group, 20% of 10-19, 11% of 20-29, 12% of 30-39, and 19% of those greater than 40 years. Of the 105 samples examined for *Amphimerus* eggs, 27 (25.7%) (95% confidence interval (CI): 17.5–34%) were positive when examined by KK technique, 22 (21%) (95% CI: 13.1–28.7) by the spontaneous sedimentation method, 19 (18%) (95% CI: 10.7–25.3) using the formalin-ether concentration method, and 1 (1%) (95% CI: 0.9–2.9) based on direct smear microscopy. The combined results of the four techniques used, showed an *Amphimerus* infection prevalence rate of 36.2% with 38 of the 105 stool samples positive. There was no significant difference in the infection rate by sex or age groups. The occurrence and prevalence of soil transmitted helminths (STH) will be reported elsewhere.

The sensitivity of *Amphimerus* eggs detection as determined by each method or combination of methods, is shown in **table 1**. Comparing each method separately the KK, SSTT and FEC provided the higher sensitivities, however there was no statistically significant difference between them ($P < 0.05$). KK technique showed the highest sensitivity (71%). However, when the 3 methods were compared with DM there was a significantly higher difference ($P > 0.01$). Combination of KK with either SSTT, or FEC had a higher sensitivity in detecting eggs than any one method used alone.

The typical characteristics of *Amphimerus* eggs observed under microscopy utilizing the four methods are showed in **Figure 1**. The eggs observed by light microscopic were oval or piriform-shaped, with a thick yellow-brown shell surrounding it, operculate, measuring 26-33 µm x 13-16 µm, with a small knob seen on the abopercular end, showing the developed miracidium takes up the interior of the eggs (Figure 1A). However, in the KK technique, the interior miracidium disappear and the membrane became thin and transparent, some were difficult to recognize (Figure 1B).

TABLE 1. **Sensitivity of *Amphimerus* eggs detection by a single and combination of methods when the combined results of four methods are considered the "gold" standard.**

Methods	n (%)	95% Confidence Interval
KK	27 (71)	57 – 85
SSTT	22 (57.9)	42.3 – 73.5
FEC	19 (50)	34.2 – 65.8
DM	01 (2.6)	-2.4 – 7.6
KK + SSTT	35 (92.1)	83.6 – 100
KK + FEC	31 (81.6)	69.3 – 93.9
KK + DM	28 (73.7)	59.8 – 87.6
SSTT + FEC	27 (71)	57 – 85
SSTT + DM	27 (71)	57 – 85
FEC + DM	20 (52.7)	36.9 – 68.5
All ("gold" standard)	38 (100)	

KK= Kato-Katz, SSTT= spontaneous sedimentation, FEC= formalin-ether concentration, DM= direct smear microscopy.

The sensitivity and negative predictive values of the individual methods compared with the combined results, which was considered our diagnostic "gold" standard, are shown in **table 2**.

TABLE 2. Sensitivity and negative predictive values (NPV) of the methods used when considering the combined results as the diagnostic "gold" standard.

Technique	Sensitivity	NPV
Kato Katz	71% (CI 95%: 66 – 75%)	85% (CI 95%: 79 – 91%)
Spontaneous Sedimentation	57.9% (CI 95%: 48.9 – 66.9%)	80% (CI 95%: 73 – 87%)
Formalin-ether	50% (CI 95%: 41 – 59%)	77% (CI 95%: 69 – 85%)
Direct Microscopy	2.6% (CI 95%: 0.4 – 5.6%)	64% (CI 95%: 55 – 73%)

TABLE 3. Kappa index and its interpretation on the methods used when considering the combined results as the diagnostic "gold" standard.

Technique	Kappa Índex– Interpretation	p-value
Kato Katz	0.758 – Good concordance	0.0001
Spontaneous Sedimentation	0.637 - Good concordance	0.0001
Formalin-ether	0.561 - Moderate concordance	0.0001
Direct Microscopy	0.033 – Very low concordance	0.182

DISCUSSION

This is the first study to report the sensitivity on *Amphimerus* spp. egg detection by the KK technique in comparison with three other coproparasitological methods. Our results demonstrated that using a non-duplicated KK method showed the higher sensitivity, identifying 27 (71%) of the 38 stool samples confirmed as *Amphimerus* positive by the four combined methods. For the second and third ranking, the spontaneous sedimentation technique in tube (SSTT) and the formalin-ether concentration (FEC) found 22 (58%) and 19 (50%) of the samples to be positive, respectively. However, only 1 (2.6%) sample was detected positive by the direct smear microscopy method (DM), this presents a great concern particularly in Ecuador where DM is the only method provided by private and public laboratories of the Ministry of Public Health (MSP).

At the moment, there is no "gold" standard diagnosis test for *Amphimerus* liver fluke, even using a ELISA and LAMP methods where sensitivity reached only to 85% and 76.6%, and specificity to 71% and 80.7%, respectively (Cevallos et al. 2017; Cevallos et al. 2017b). Here, we used the combined results of the 4 different methods as the diagnostic "gold" standard for *Amphimerus* infection as has been used in previous studies for STH (Santos et al. 2005, Goodman et al, 2007, Steinmann et al. 2008, Utzinger et al. 2008). Summing together the results of the 4 methods employed, a high prevalence rate of 36.2% for *Amphimerus* infection was found in the Chachi community studied. Merging KK and SSTT results, the sensitivity raised to 92.1% (35 positive samples) and to 81.6% (31 samples) by combining KK and FEC methods. Therefore, performing two techniques on a single sample enhanced the detection of *Amphimerus* infection.

In the present study, SSTT detected 9 extra positive samples that were negative with KK, enhancing the diagnosis by 25% as compared with KK. This observation might be explained for that with the SSTT, 5 g of fecal matter are used as compared to 41,7 mg for KK. SSTT has the advantage of not needing expensive reagents; just warm saline and two 50-mL plastic tubes that could be recycled after careful washing. On the contrary, KK requires 3% malachite-green glycerol or 3% methylene blue-glycerol solution, and FEC method needs 10% buffered-formalin and centrifuge equipment. SSTT had already been used in several studies for diagnosis of intestinal parasites in developing countries and showed to be highly effective and inexpensive (Tello et al. 2012). For this study KK kits were purchased for $400 USD expecting to do 400 stool examinations.

According to Tello et al (2012) the cost for SSTT is approximately $0.03 USD per sample. Another important advantage of the SSTT method as compared to KK in field work setting, is that eggs of Opisthorchiidae members are hard and are not easily broken. Therefore, a stool sample can be fixed, transported, and preserved in 10% formalin, merthiolate-iodine-formalin (MIF) or sodium acetate-acetic acid-formalin (SAF) solutions for several days before SSTT is performed whilst with the KK method, examination needs to be processed using fresh stool samples and observed the same day. Hence, based on our field experience for detecting *Amphimerus* infection, we would

recommend SSTT because this method is easy to perform in field conditions, cost-effective and capable for high volume screening of large populations.

Low sensitivities for DM has been reported elsewhere (WHO 2002, Goodman et al. 2007, Keiser & Utzinger, 2009, Endris et al. 2013, Tello et al. 2012) as well as in our previous study for *Amphimerus* (Calvopina et al. 2011). Unlike KK, SSTT and FEC, very small amount of fecal material is processed for DM, approximately 2 mg, which could explain, in part, its low sensitivity. Given the low sensitivity of DM and therefore the high probability of missing *Amphimerus* infections, the utilization of this technique should be discouraged for amphimeriasis diagnosis, especially when used alone.

The observed prevalence of *Amphimerus* infection based on the KK technique was higher in comparison with the other 3 methods. However, there was no significant difference statistically. In contrast, comparing the KK, SSTT and FEC methods against DM, a significant difference was found. It is also corroborated by the Kappa index where both KK and SSTT has good concordance whilst moderate and very low concordance by FEC and DM, respectively. Examination of duplicate 41.7-mg stool sample and/or multiple KK thick smear from one or 2 stool samples and in different days could enhance the sensitivity of *Amphimerus* diagnosis, but it would increase labor, costs, and be more time consuming (Utzinger et al. 2008, Goodman et al. 2007). Furthermore, processing multiple samples for KK under field-work conditions, with limited resources such as power supply, as we did in the present study, would be challenging. It is noteworthy to mention that using the KK method, *Amphimerus* eggs were not detected in 11 (29%) stool specimens that were observed using the other 3 methods, this can be explained because the *Amphimerus* eggs can became transparent, thin shelled and could be easily missed as is showed in figure 1B.

Also they can even disappear due to glycerin when long delays occur between the preparation and the microscopic observation. A previous study showed that using duplicate slides for KK, the sensitivity for detecting STH increased from 74 to 95% at high infection intensity, however, dropped to 53-80% in low intensity settings (Nikolay 2014). In our study, existed a positive correlation between the intensity of infection, based on fecal egg counts demonstrated by KK technique, with the detection of eggs observed by SSTT and FEC methods (data not shown). A disadvantage for FEC method is that reagents used may be hazardous or can be irritant; ether is highly flammable. The sedimentation technique used at CDC is the formalin-ethyl acetate technique, a diphasic sedimentation technique that avoids the problems of flammability of ether, however this technique needs a centrifuge and is time-consuming.

Methods requiring a centrifuge as FLOTAC have a distinct disadvantage in field laboratory settings, and also involve more procedural steps. The Mini-FLOTAC needs a closed chamber for flotation and mixing, and a separate reading disc, all resulting in higher costs and procedures. McMaster method is a flotation technique and therefore does

not detect eggs of trematodes as is *Amphimerus* or *Schistosoma mansoni* (Assefa et al. 2014).

Additionally, our study confirms the high prevalence of human infection by *Amphimerus* in the Chachi group, who reside in the tropical rain forest along the Cayapas river, in the province of Esmeraldas, Ecuador where the first human infections were described (Calvopina et al. 2011). The prevalence of infection in this study (36.2%) is much higher compared to that previously reported of 24% (Calvopina et al. 2011). In the previous study, the most remote village (120 km inland from the coast) had the highest prevalence. In the present report the study community was 135 km inland from the coast, suggesting that the villagers living alongside the upper tributaries of the Cayapas river, in this area, are more infected.

It is important to note that trematode species-specific diagnosis based on egg morphology poses a problem for liver fluke of the Opisthorchiidae and for minute intestinal flukes of Heterophyidae families (Kaewkes 2003). The size and shapes of eggs of the members of the above mentioned families are quite similar to *Amphimerus* eggs (Calvopina et al. 2011 and 2015). However, it should not be a concern in South American countries, including Ecuador, because those liver and intestinal flukes are only found in Asian countries (see below) and has not been reported in humans of the Americas (Keiser and Utzinger, 2009). In addition, definitive diagnosis of eggs found was confirmed to be *Amphimerus* by scanning electronic microscopy (SEM) and examination of adult worms obtained from the bile ducts of humans, cats and dogs in the same area where this study was performed (Calvopina et al. 2011 and 2015).

In conclusion, the routinely used laboratory technique of DM method will underestimate the prevalence of *Amphimerus* infection. Therefore, the use of additional diagnostic methods is mandatory. The KK technique detected the greatest number of positive samples for eggs of *Amphimerus*. However, 11 (29%) additional samples were detected positive by SSTT and FEC, that were missed by KK method. Our results indicate that SSTT would be the more cost effectiveness method in field conditions and the screening a stool sample by 2 or 3 methods is likely to detect more *Amphimerus* infections in endemic communities.

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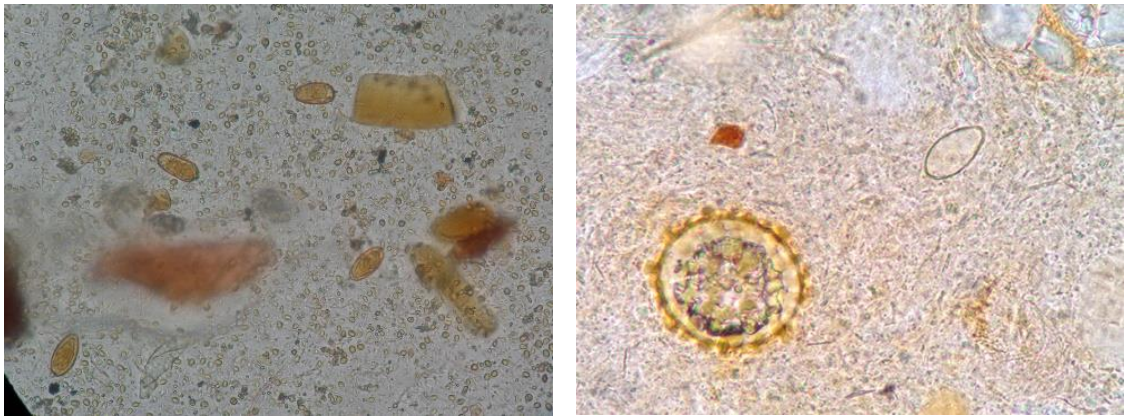

FIGURE 1. **A.** Four eggs of *Amphimerus* spp. in an Lugol´s stained wet mount of concentrated stool (200x magnification). **B.** An egg of *Amphimerus* spp. observed in a slide by the Kato-Katz method (400x) in comparison with a fertile *Ascaris lumbricoides* egg.

6. ANEXOS

Anexo 1. Metodología

1. Obtención del parásito adulto.

Los parásitos adultos de *Amphimerus* spp. fueron obtenidos del hígado de un gato de la zona endémica del Río Cayapas, previo a una eutanasia del animal y al consentimiento informado de sus dueños. El hígado fue lavado con agua destilada y posteriormente depositado en un recipiente con NaCl al 0,9% (Figura 14). Se realizaron varios cortes transversales al eje horizontal, se recuperaron parásitos adultos durante 2 h y a continuación fueron colocados en tubos tipo Falcon de 15 mL conteniendo PBS estéril.

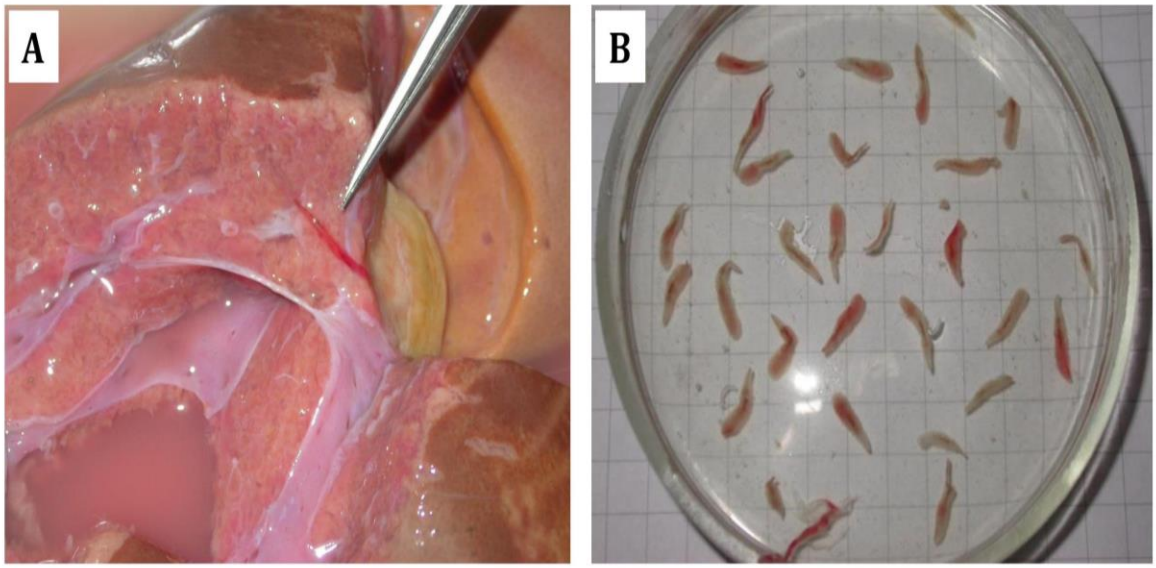

Figura 14. Obtención de parásito *Amphimerus* spp. adulto.

A. Hígado de gato. **B.** Lavado con agua estéril para posterior conservación del parásito.

2. Preparación de antígeno somático de vermes adultos de *Amphimerus* spp.

Se realizó directamente en el campo a partir de parásitos adultos de *Amphimerus* spp. que fueron lavados tres veces con PBS estéril (pH 7,4) temperatura de a 4°C en una cámara pequeña con hielo seco. Se colocaron 90 parásitos en tubos de 15 mL (Falcon ®) añadiendo 5 mL de tampón de lisis que contiene Buffer Tris-base10mM-EDTA1mM y 500 µL de una mezcla de inhibidores de proteasas (UltraCruz® Protease Inhibitor Cocktail Tablet). Los parásitos fueron congelados a -20°C hasta el siguiente día. Posteriormente, fueron homogenizados completamente con la ayuda de un sonicador (ultrasonic processor). La sonicación se realizó en 4 ciclos con 60% de amplitud cada ciclo con una duración de 1 min y 3 min de descanso. La solución generada en el sonicado se centrifugó 2 veces durante 15 min a 3.500 rpm a 4 °C.

El sobrenadante fue dializado utilizando una membrana de diálisis (Spectra/Por® Dialysis MWCO: 12-14,000). Se utilizó agua destilada estéril (H_2O_d) y 0.01% de mertiolate blanco. La diálisis se realizó durante 72 h con cambios de agua cada 24 h a 4°C.

Para concentrar el antígeno dializado se realizó una segunda diálisis de saturación con sucrosa en polvo, colocando la membrana en un recipiente y cubriéndolo totalmente con sucrosa durante 5 h. El producto se almacenó a -40°C en alícuotas de 10 µL hasta su utilización como antígeno crudo total soluble de *Amphimerus* spp.

El antígeno obtenido fue liofilizado y almacenado en tubos Nunc™ a -20 °C hasta la realización de la prueba ELISA. En el CIETUS (Universidad de Salamanca, Salamanca, España), una alícuota del antígeno fue reconstituida en 600 µL de PBS estéril. La cuantificación de la concentración de antígeno se valoró mediante colorimetría, utilizando Albúmina Sérica Bovina (BSA) como proteína de referencia. Se determinó una concentración de 0,21 µg/µL.

3. Desarrollo de la técnica de ELISA.

Para el desarrollo de la técnica ELISA, los antígenos fueron conservados en todo momento en alícuotas de 100 µL a -20 °C. Tras varios ensayos de estandarización, el protocolo final aprobado fue el siguiente: *(1)* Tapizado de la placa de poliestireno de fondo plano con 100 µL de antígeno somático de *Amphimerus* spp., a razón de 4 µg/mL diluido en tampón carbonato pH 9,6. Se cubrió y se dejó incubar toda la noche (12-18 h) a 4 °C. *(2)* Se diluyeron los sueros en PBS-Tween y se incubaron a 4 °C. *(3)* Al siguiente día se realizó el bloqueo de la placa con BSA al 2% (PBS-Tween). La primera placa de 96 pocillos se preparó en 4 mL de PBS + 0,08 g de BSA. Se colocó 100 µL por pocillo y se incubó a 37 °C durante 1 h. *(4)* Lavado de la placa con PBS-Tween 20 al 0,05% durante 3 min. Se repite esta operación 3 veces. Se colocó 200 µL por pocillo. *(5)* Se

añadió el suero problema a la dilución de 1:50 en PBS-Tween. 100 µL por pocillo. Se cubrió la placa y se incubó a 37 ºC durante 1 h. *(6)* Lavado de la placa con PBS-Tween 20 al 0,05% durante 3 minutos. Se repite esta operación por 3 ocasiones. Se colocó 200 µL por pocillo. *(7)* Se añadió el conjugado o anticuerpo secundario (Sigma), marcado con la enzima peroxidasa a una dilución 1:2000 en PBS-Tween, 100 µL por pocillo. Se incubó la placa durante 1 h a 37 ºC. *(8)* Lavado de la placa con PBS-Tween 20 al 0,05% durante 3 min. Se repite esta operación tres veces. Se colocó 200 µL por pocillo. *(9)* Posteriormente, se reveló la reacción utilizando solución de revelado ($C_6H_8O_7$, PO_4HNa_2, H_2O y OPD), se colocó 100 µL/pocillo. Incubar en oscuridad durante 2-10 min. Es importante que el agua oxigenada se añada al final de la preparación de la solución de revelado. Finalmente se paró la reacción con 50 µL/pocillo de H_2SO_4 3N y se leyó la densidad óptica (DO) en un lector de ELISA (Ear400FT ELISA reader Lab. Instruments), utilizando un filtro de longitud de onda de 492 nm.

Materiales y reactivos

1. Péptido sintético o extracto del parásito (antígeno).
2. Sueros humanos (anticuerpo primario).
3. Anticuerpo secundario (IgG anti-humano) acoplados a peroxidasa.
4. Tampón Fosfato Salino al 5%; pH 7,20: Na_2HPO_4 (1,07g), NaH_2PO_4 (0,39g), NaCl (8,5g), H_2O (1L), Tween 20 (500 µL).
5. Tampón Carbonato. pH 9,6: Na_2CO_3 (0,159 g), $NaHCO_3$ (0,293 g), H_2O (100 mL).
6. Tampón Citrato. pH 5: $C_6H_8O_7$ (2,14 g), PO_4HNa_2 (1,4 g), H_2O (400 mL).
7. Ácido Sulfúrico. 3N: H_2SO_4 (8mL), H_2O (92mL).
8. Solución de Revelado: Tampón Citrato (20 mL) y OPD (0,0053 g). Para la placa de 96 pocillos se utilizaron 0,0028 g en 10 mL H_2O_2 (8 µL). Éste se añade justo antes de colocarlo en los pocillos.

4. Desarrollo de la TD-PCR.

Para comprobar si el anillamiento de los cebadores externos F3 y B3 generaba una amplificación del tamaño esperado *in silico* según el diseño previo informático (209 pb), se realizó una *touchdown* PCR (TD-PCR). En esta variante de la PCR la temperatura de anillamiento va disminuyendo en cada ciclo de amplificación de manera que garantiza un abanico de temperaturas de anillamiento de los cebadores idóneo para la correcta amplificación de la secuencia diana. Se ensayaron diferentes condiciones. En la tabla 5,

se muestran las concentraciones finalmente utilizadas para la amplificación y los reactivos usados para la mezcla de la reacción. Todas las reacciones de PCR se llevaron a cabo en un termociclador *Mastercycler Gradient®-96 well* (Eppendorf).

Para determinar la sensibilidad de la PCR (F3-B3) se utilizaron diluciones seriadas desde 1×10^{-1} a 1×10^{-9} ng/µL de ADN de *Amphimerus* spp. las cuales fueron preparadas con agua ultrapura y almacenadaa a -20 ^{o}C. Todos los ensayos de la PCR se realizaron con 2 µL de ADN diana (5 ng/µL) en cada caso. Siempre se incluyeron controles negativos (agua ultrapura) y controles positivos (ADN genómico de *Amphimerus* spp.). Para valorar la especificidad se usaron muestras de ADN de otros parásitos disponibles en el CIETUS y también de *Clonorchis sinensis* y *Opisthorchis viverrini* (0,5 ng/µL). En todos los casos se utilizó 2 µL de ADN molde y en cada reacción se incluyeron controles negativos a (con agua ultrapura en lugar de ADN) y controles positivos (con ADN de *Amphimerus* spp.)

Tabla 5. Componentes y condiciones de reacción de la PCR (F3-B3).

Mezcla de reacción y condiciones de amplificación utilizadas en la TD-PCR con los cebadores externos F3 y B3 para la amplificación de la secuencia nucleotídica de 209 pb de ADN de *Amphimerus* spp.

Componentes	**Volumen**
H_20	15.1 µL
10X Buffer	2.5 µL
$MgCl_2$ (25 mM)	1.5 µL
dNTPs (2.5 mM)	2.5 µL
F3 (5 pmol)	0.5 µL
B3 (5 pmol)	0.5 µL
Taq-polimerasa (2 U)	0.4 µL
ADN molde	2 µL
TOTAL	**25 µL**

Tª (°C)	**Tiempo**	**Ciclos**
94	1 min	x 1
94	20 s	x 2
64-58	20 s	
72	30 s	
94	1 min	
94	1 min	x 15
57	20 s	
72	30 s	
72	10 min	x 1

5. Desarrollo de la técnica LAMPhimerus.

5.1 Obtención de ADN de *Amphimerus* spp.

Para la obtención del ADN genómico de *Amphimerus* spp. se utilizó un total de 100 parásitos adultos obtenidos de pacientes de las comunidades de Corriente Seca y Estero Vicente del Río Cayapas. Los ejemplares adultos fueron recuperados tras el tratamiento de los pacientes con praziquantel y mantenidos en alcohol hasta la extracción del ADN en el laboratorio del CIETUS mediante el kit comercial *G-spin Total DNA Extraction Kit* (Intron Biotechnology) siguiendo las instrucciones del fabricante. El ADN obtenido se cuantificó mediante espectrofotometría utilizando un Nanodrop (ND-1000) y se diluyó en agua ultrapura a concentraciones finales de 5 ng/µL y 0.5 ng/µL; a partir de estas concentraciones se realizaron diluciones seriadas desde 1×10^{-1} a 1×10^{-9} ng/µL. Este ADN se utilizó como control positivo en todas las reacciones de PCR y LAMP realizadas y también para evaluar la sensibilidad de las dos técnicas moleculares.

5.2 Obtención de ADN de otros parásitos.

Para evaluar la especificidad de la PCR y de la técnica LAMP se utilizó un total de 16 muestras de ADN de otros parásitos disponibles en el laboratorio del CIETUS, incluyendo varios helmintos y protozoos. Los ADN utilizados fueron de los siguientes parásitos: *Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma mansoni, S. haematobium, S. japonicum, S. intercalatum, Fasciola hepatica, Dicrocoelium dendriticum, Onchocerca volvulus, Strongyloides venezuelensis, Trichinella spiralis, Taenia truncata, Echinococcus granulosus, Cryptosporidium parvum, Giardia duodenalis y Entamoeba histolytica.* La concentración utilizada para cada muestra fue de 0.5 ng/µL.

5.3 Diseño del LAMP

Tras la búsqueda de secuencias disponibles en las bases de datos de *GenBank* (http://www.ncbi.nlm.nih.gov) y comparación mediante BLAST (Altschul et al., 1990), se seleccionó una diana de amplificación correspondiente a una secuencia de 459 pb de ADN lineal de *Amphimerus* spp. HS-2011 aislado de hospedador humano (Genbank accesion no. AB678442.1) (Calvopiña et al., 2015) sobre la que se diseñaron los cebadores (F3, B3, FIP y BIP) utilizando el software *Primer Explorer V4* (https://primerexplorer.jp/e). Inicialmente, el software generó varios juegos de 4 cebadores; se seleccionó el más apropiado en función de los criterios descritos en el "Manual del software para la generación de cebadores para LAMP" (http://primerexplorer.jp/e/v4_manual/index.html). Una vez seleccionados, su síntesis se encargó a la empresa Fisher Scientific y se resuspendieron en agua ultrapura a una concentración final de 100 pmol/µL. El juego de cebadores se probó con diferentes mezclas de reacción empleando distintas enzimas (*Bst* ADN polimerasa

Large Fragment, *Bst* ADN polimerasa 2.0 y *Bst* ADN polimerasa 2.0 *Warm Start*) y concentraciones variables de $MgSO_4$ y betaína a distintas temperaturas de incubación (61°C, 63 °C y 65 °C). Finalmente, se seleccionó la temperatura de 63 °C como más idónea para la amplificación utilizando la mezcla de reacción que aparece en la tabla 6.

Tabla 6. Componentes utilizados en la reacción LAMPhimerus

Mezcla de reacción utilizada en la reacción LAMPhimerus. La enzima *Bst* polimerasa 2.0 WarmStart, buffer y $MgSO_4$ fueron suministrados por New England Biolabs; la betaína por SIGMA y los dNTPs por Intron.

Componentes	**Volumen (µL)**
H_2O	7,7
Betaína (1M)	5
$MgSO_4$ (0.8 mM)	1,5
dNTPs (2.5 mM)	3,5
10X Buffer	2,5
FIP (40 pmol/µL)	0,4
BIP (40 pmol/µL)	0,4
F3 (5 pmol/µL)	0,5
B3 (5pmol/µL)	0,5
Bst polimerasa 2.0 Warm Start	1
ADN	2
TOTAL	**25**

6. Método LAMP para la amplificación de ADN de *Amphimerus* spp.

Para comprobar el correcto funcionamiento del juego de 4 cebadores sintetizados, se realizaron diversas reacciones de amplificación utilizando ADN de *Amphimerus* spp. como control positivo de la reacción. Todas las reacciones se incubaron en un termobloque K Dry-Bath® (No. DB-006) a 63 ^{o}C durante 60 min o 120 min; transcurrido ese tiempo, la temperatura se elevó a 80 ^{o}C durante 10 min para desactivar a la enzima y detener la reacción.

Para valorar la especificidad del LAMPhimerus se utilizaron las muestras de ADN de los distintos parásitos mencionados anteriormente. El límite de detección de la técnica se estableció con las diluciones seriadas (10^{-1} a 10^{-9} ng/µL) obtenidas a partir de ADN de gusanos adultos de *Amphimerus* spp.

7. Detección de los productos de amplificación.

7.1 TD-PCR.

Para la detección de los productos obtenidos mediante PCR se utilizó la electroforesis en gel de agarosa al 1,5% (100 mL de TBE, 1,5 g de agarosa y 5 µL de bromuro de etidio) a 60v durante 20 min y posteriormente a 100v durante aproximadamente 1h. Los geles se visualizaron utilizando un transiluminador con luz UV (*Gel documentation system*, UVItec, UK). Por último, los geles se fotografiaron y almacenaron en un soporte informático para su manejo y edición.

7.2 LAMP.

En el caso de la amplificación mediante la técnica LAMP, la visualización de los resultados se realizó mediante:

A. Colorimétricamente. Se añadió a los tubos de reacción 2 µL (dilución 1:10; 10.000X) del colorante fluorescente SYBR Green I (Invitrogen™). En los tubos en los que hubo amplificación se produjo un viraje del colorante de naranja a verde. En los tubos negativos se mantuvo el color naranja original propio del colorante.

B. Electroforesis en gel de agarosa. De igual forma que para la detección de los productos obtenidos mediante TD-PCR.

8. Análisis estadísticos

Para estimar la exactitud los métodos de ELISA y LAMPhimerus como pruebas diagnósticas se calcularon los porcentajes de sensibilidad, especificidad, valor predictivo positivo (VPP) y valor predictivo negativo (VPN); también la curva ROC (*Receiver Operating Characteristic* o Característica Operativa del Receptos) para el ELISA, utilizando los programas estadísticos SPSS v.22 (disponible en https://www.ibm.com) y MedCalc v. 15.2.2 (MedCalc Software, Ostende, Belgium; www.medcalc.org).

Anexo 2. Otras publicaciones con índice de impacto

Determinants of Short-term Movement in a Developing Region and Implications for Disease Transmission.

Kraay ANM, Trostle J, Brouwer AF, Cevallos W, Eisenberg JNS.

Epidemiology. 2018 Jan;29(1):117-125.

Intralesional Infiltration with Meglumine Antimoniate for the Treatment of Leishmaniasis Recidiva Cutis in Ecuador.

Calvopiña M, Cevallos W, Paredes Y, Puebla E, Flores J, Loor R, Padilla J.

Am J Trop Med Hyg. 2017 Nov;97(5):1508-1512.

Coinfection of Leishmania guyanensis and Human Immunodeficiency Virus-Acquired Immune Deficiency Syndrome: Report of a Case of Disseminated Cutaneous Leishmaniasis in Ecuador.

Calvopina M, Aguirre C, Cevallos W, Castillo A, Abbasi I, Warburg A.

Am J Trop Med Hyg. 2017 May;96(5):1151-1154.

I get height with a little help from my friends: herd protection from sanitation on child growth in rural Ecuador.

Fuller JA, Villamor E, Cevallos W, Trostle J, Eisenberg JN.

Int J Epidemiol. 2016 Apr;45(2):460-9.

Antibiotic Resistance in Animal and Environmental Samples Associated with Small-ScalePoultry Farming in Northwestern Ecuador.

Braykov NP, Eisenberg JN, Grossman M, Zhang L, Vasco K, Cevallos W, Muñoz D, Acevedo A, Moser KA, Marrs CF, Foxman B, Trostle J, Trueba G, Levy K.

mSphere. 2016 Feb 10;1(1). pii: e00021-15.

Distribution of Enteroinvasive and Enterotoxigenic Escherichia coli Across Space and Time in Northwestern Ecuador.

Bhavnani D, Bayas Rde L, Lopez VK, Zhang L, Trueba G, Foxman B, Marrs C, Cevallos W, Eisenberg JN.

Am J Trop Med Hyg. 2016 Feb;94(2):276-84.

Association of Household Food Insecurity with the Mental and Physical Health of Low-Income Urban Ecuadorian Women with Children.

Weigel MM, Armijos RX, Racines M, Cevallos W, Castro NP.

J Environ Public Health. 2016 Sep 26. PubMed PMID: 27752266.

Food Insecurity Is Associated with Undernutrition but Not Overnutrition in Ecuadorian Women from Low-Income Urban Neighborhoods.

Weigel MM, Armijos RX, Racines M, Cevallos W.

J Environ Public Health. Epub 2016 Mar 23. PubMed PMID: 27110253.

Effects of selection pressure and genetic association on the relationship between antibiotic resistance and virulence in *Escherichia coli*.

Zhang L, Levy K, Trueba G, Cevallos W, Trostle J, Foxman B, Marrs CF, Eisenberg JN.

Antimicrob Agents Chemother. 2015 Nov; 59 (11): 6733-40.

Unexpected distribution of the fluoroquinolone-resistance gene qnrB in Escherichia coli isolates from different human and poultry origins in Ecuador.

Armas-Freire PI, Trueba G, Proaño-Bolaños C, Levy K, Zhang L, Marrs CF, Cevallos W, Eisenberg JN.

Int Microbiol. 2015 Jun;18 (2): 85-90.

A national survey to determine prevalence of *Trypanosoma cruzi* infection among pregnant women in Ecuador.

Costales JA, Sánchez-Gómez A, Silva-Aycaguer LC, Cevallos W, Tamayo S, Yumiseva CA, Jacobson JO, Martini L, Carrera CA, Grijalva MJ.

Am J Trop Med Hyg. 2015 Apr; 92 (4): 807-10.

Spatial Variability of *Escherichia coli* in Rivers of Northern Coastal Ecuador.

Rao G, Eisenberg JN, Kleinbaum DG, Cevallos W, Trueba G, Levy K.

Water (Basel). 2015 Feb 13;7 (2): 818-832.

Identifying etiological agents causing diarrhea in low income Ecuadorian communities.

Vasco G, Trueba G, Atherton R, Calvopiña M, Cevallos W, Andrade T, Eguiguren M, Eisenberg JN.

Am J Trop Med Hyg. 2014 Sep; 91(3): 563-9.

Household effectiveness vs. laboratory efficacy of point-of-use chlorination.

Levy K, Anderson L, Robb KA, Cevallos W, Trueba G, Eisenberg JN.

Water Res. 2014 May 1; 54: 69-77.

Impact of rainfall on diarrheal disease risk associated with unimproved water and sanitation.

Bhavnani D, Goldstick JE, Cevallos W, Trueba G, Eisenberg JN.

Am J Trop Med Hyg. 2014 Apr; 90(4): 705-11.

Heavy rainfall events and diarrhea incidence: the role of social and environmental factors.

Carlton EJ, Eisenberg JN, Goldstick J, Cevallos W, Trostle J, Levy K.

Am J Epidemiol. 2014 Feb 1; 179 (3): 344-52.

HIV and syphilis infection in pregnant women in Ecuador: prevalence and characteristics of antenatal care.

Sánchez-Gómez A, Grijalva MJ, Silva-Aycaguer LC, Tamayo S, Yumiseva CA, Costales JA, Jacobson JO, Chiriboga M, Champutiz E, Mosquera C, Larrea M, Cevallos W.

Sex Transm Infect. 2014 Feb; 90(1): 70-5.

Molecular identification of *Giardia duodenalis* in Ecuador by polymerase chain reaction-restriction fragment length polymorphism.

Atherton R, Bhavnani D, Calvopiña M, Vicuña Y, Cevallos W, Eisenberg J.

Mem Inst Oswaldo Cruz. 2013 Jun; 108 (4): 512-5.

Social connectedness and disease transmission: social organization, cohesion, village context, and infection risk in rural Ecuador.

Zelner JL, Trostle J, Goldstick JE, Cevallos W, House JS, Eisenberg JN

Am J Public Health. 2012 Dec; 102 (12): 2233-9.

Synergistic effects between rotavirus and coinfecting pathogens on diarrheal disease: evidence from a community-based study in northwestern Ecuador.

Bhavnani D, Goldstick JE, Cevallos W, Trueba G, Eisenberg JN.

Am J Epidemiol. 2012 Sep 1; 176 (5): 387-95.

In-roads to the spread of antibiotic resistance: regional patterns of microbial transmission in northern coastal Ecuador.

Eisenberg JN, Goldstick J, Cevallos W, Trueba G, Levy K, Scott J, Percha B, Segovia R, Ponce K, Hubbard A, Marrs C, Foxman B, Smith DL, Trostle J.

J R Soc Interface. 2012 May 7; 9 (70): 1029-39.

Where science meets policy: comparing longitudinal and cross-sectional designs to address diarrhoeal disease burden in the developing world.

Markovitz AR, Goldstick JE, Levy K, Cevallos W, Mukherjee B, Trostle JA, Eisenberg JN.

Int J Epidemiol. 2012 Apr; 41 (2): 504-13.

***Plesiomonas shigelloides* infection, Ecuador, 2004-2008.**

Escobar JC, Bhavnani D, Trueba G, Ponce K, Cevallos W, Eisenberg J.

Emerg Infect Dis. 2012 Feb;18 (2): 322-4.

Rapid changes in rotaviral genotypes in Ecuador.

Hasing ME, Trueba G, Baquero MI, Ponce K, Cevallos W, Solberg OD, Eisenberg JN.

J Med Virol. 2009 Dec; 81 (12): 2109-13.

Raising the level of analysis of food-borne outbreaks: food-sharing networks in rural coastal Ecuador.

Trostle JA, Hubbard A, Scott J, Cevallos W, Bates SJ, Eisenberg JN.

Epidemiology. 2008 May; 19 (3): 384-90.

Relating diarrheal disease to social networks and the geographic configuration of communities in rural Ecuador.

Bates SJ, Trostle J, Cevallos WT, Hubbard A, Eisenberg JN.

Am J Epidemiol. 2007 Nov 1; 166 (9): 1088-95.

Symptomatic and subclinical infection with rotavirus P[8]G9, rural Ecuador.

Endara P, Trueba G, Solberg OD, Bates SJ, Ponce K, Cevallos W, Matthijnssens J, Eisenberg JN.

Emerg Infect Dis. 2007 Apr; 13(4): 574-80.

High prevalence of enteroinvasive *Escherichia coli* isolated in a remote region of northern coastal Ecuador.

Vieira N, Bates SJ, Solberg OD, Ponce K, Howsmon R, Cevallos W, Trueba G, Riley L, Eisenberg JN.

Am J Trop Med Hyg. 2007 Mar; 76 (3): 528-33.

Environmental change and infectious disease: how new roads affect the transmission of diarrheal pathogens in rural Ecuador.

Eisenberg JN, Cevallos W, Ponce K, Levy K, Bates SJ, Scott JC, Hubbard A, Vieira N, Endara P, Espinel M, Trueba G, Riley LW, Trostle J.

Proc Natl Acad Sci U S A. 2006 Dec 19; 103 (51): 19460-5.

Anexo 3. Contribuciones en Congresos

Divulgación científica de resultados ELISA, PCR y LAMPhimerus.

1ER CONGRESO DE INMUNOLOGÍA EN ENFERMEDADES INFECCIOSAS Y PARASITARIAS

CERTIFICADO

Otorgado a:

MSc. William Cevallos Trujillo

Por su participación en calidad de Expositor de Póster Científico en el **I CONGRESO NACIONAL DE INMUNOLOGIA EN ENFERMEDADES INFECCIOSAS Y PARASITARIAS,** realizado en la ciudad de Quito del 1 al 4 de diciembre del 2015.

Ing. Edison Apunte Castillo. DIRECTOR EJECUTIVO INSPI

Ec. Lissette Gavilanes DIRECTORA DE INVESTIGACIÓN, DESARROLLO E INNOVACIÓN - INSPI

Dra. Ana Lucía Ruano DIRECTORA PROGRAMA - PROPAD - INSPI

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La Pontificia Universidad Católica del Ecuador a través de la Facultad de Ciencias Exactas y Naturales y su Centro de Investigación para la Salud en América Latina (CISeAL)

CERTIFICAN QUE:

William Cevallos

Presentó el trabajo titulado:

Diagnóstico inmunológico y molecular de la amphimeriasis- **SIMPOSIO**

en el IV Encuentro Internacional de Investigación en Enfermedades Infecciosas y Medicina Tropical que se llevó a cabo del 13 al 15 de junio de 2016 en las instalaciones de la PUCE, Quito - Ecuador

QUITO, 19 DE JUNIO DE 2016

Dr. Mario Grijalva Cobo
Director CISeAL PUCE

Dra. Ana Lucía Moncayo
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EN LABORATORIO CLÍNICO

Confiere el presente

CERTIFICADO

Al (a) Dr. William Cevallos, Msc.

Por su participación en calidad de Conferencista

EN EL IV CONGRESO LATINOAMERICANO DE ACTUALIZACIÓN Y EDUCACIÓN CONTINUA EN MEDICINA DE LABORATORIO Y TRASLACIONAL

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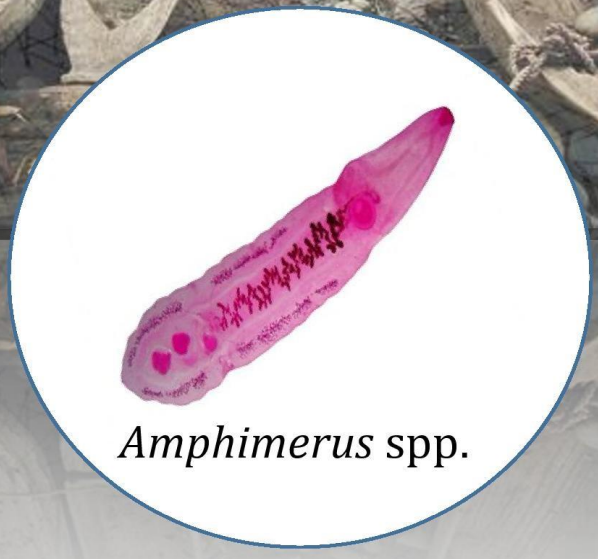

Amphimerus spp.

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